AMERICAN FERN JOURNAL

Volume 100

Number 1

January–March 2010

QUARTERLY JOURNAL OF THE AMERICAN FERN SOCIETY

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The "American Fern Journal" (ISSN 0002-8444) is an illustrated quarterly devoted to the general study of ferns. It is owned by the American Fern Society, and published at The American Fern Society, % Missouri Botanical Garden, P. O. Box 299, St. Louis, MO 63166-0299. Periodicals postage paid at St. Louis, MO, and additional entry.

Claims for missing issues, made 6 months (domestic) to 12 months (foreign) after the date of issue, and orders for back issues should be addressed to Dr. James D. Montgomery, Ecology III, 804 Salem Blvd., Berwick, PA 18603-9801.

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American Fern Journal 100(1):1–15 (2010)

Cloning and Sequence Analysis of Red/Blue Light Chimeric Photoreceptor Genes from Three Fern Species (*Coniogramme intermedia* var. *glabra*, *Plagiogyria distinctissima* and *Pronephrium lakhimpurnense*)

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ABSTRACT.—Neochrome (NEO) is a particular chimeric photoreceptor that can respond to red/far red as well as blue light. Using this photoreceptor, some cryptogams may enhance light sensitivity at low light environment. However, the *NEO* sequence information is extremely limited. Using inverse PCR and single oligonucleotide nested PCR genome-walking approaches, the full-length genomic sequences of *NEO* genes were determined for three fern species *Coniogramme intermedia* var. *glabra*, *Plagiogyria distinctissima* and *Pronephrium lakhimpurnense*. Sequence and structure analysis results indicate that they possess a conserved structure and organization. Introns are absent in three genes. They each contain an 4332, 4308 and 4317-bp ORF (Open reading frame), encoding a deduced protein with 1443, 1435 and 1438 amino acid residues. Their N-terminus consists of a putative functional phytochrome sensory module including PAS (Per / Arnt / Sim), GAF (cGMP specific phosphodiesterase / adenylate cyclases / formate hydrogen lyase transcription) and PHY (phytochrome) domains, whereas the C-terminus contains a nearly complete phototropin with two LOV (Light, Oxygen and Voltage-sensing) and one STKc (Serine/Threonine protein kinases, catalytic) domains. Phylogenetic analyses revealed that they are evolutionarily close to their homolog *AcNEO* found in *Adiantum capillus-veneris*. The 3D structures of the functional domains were computer-modeled, and the pivotal amino acid sites for each functional domain were also identified. This study represents the first comparative analysis of *NEO* nucleotide sequences and structures in ferns.

KEY WORDS.—Fern, Red/blue light chimeric photoreceptors, Sequence analysis, Structure prediction

Light is one of the most essential environmental factors for plants that perform photosynthesis. Plants have developed a variety of photoreceptors

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that perceive light signals from UV-B to far-red regions (Pepper *et al.*, 2001; Quail, 2002). Phytochromes predominantly absorb red and far-red light, using a bilin (or linear tetrapyrrole) chromophore for light detection in seed plants and in cryptogams (Quail, 2002), and phototropins are UV-A/blue light receptors (Briggs and Christie, 2002; Folta and Spalding, 2001). Many photomorphogenic responses mediated by these photoreceptors, for example, circadian clocks, shade-avoidance, phototropism, stomatal opening and chloroplast relocation, have been described (Christie, 2007; Rockwell *et al.*, 2006). Most plant species from algae to flowering plants use blue light for inducing phototropism and chloroplast movement (Campbell and Liscum, 2001; Sakai *et al.*, 2001), but some ferns and green algae use red as well as blue light for the regulation of these responses (Kagawa and Wada, 1994; Wada and Kadota, 1989). In the case of *Adiantum capillus-veneris* L., a particular chimeric photoreceptor named phytochrome 3 (later renamed as neochrome, NEO) has been identified (Nozue *et al.*, 1998; Suetsugu *et al.*, 2005), which can sense red and blue light (RL and BL) simultaneously to regulate phototropism and chloroplast movement (Kanegae *et al.*, 2006; Kawai *et al.*, 2003).

Functional domains of NEO have been characterized through biochemical, genetic, and bioinformatic approaches (Crosson and Moffat, 2001; Pandini and Bonati, 2005; Tokutomi *et al.*, 2008; Wagner *et al.*, 2005). Its N-terminus is quite similar to canonical phytochromes, whereas the C-terminus is made up of a complete phototropin motif. It has been suggested that both phytochrome and phototropin photosensory systems coexist in NEO, conferring it the potential to process red and blue light signals synergistically, which increases the light sensitivity (Kanegae *et al.*, 2006). Many polypod ferns preferentially grow in shady habitats under the canopy of dense angiosperm forests. For them, this dual-channel photoreceptor has been proposed to play a central role in developing adaptive mechanisms and promoting species diversification under low-light angiosperm forest niches (Kawai *et al.*, 2003; Schneider *et al.*, 2004).

To date, similar NEO-like chimeric photoreceptors have only been identified in a few cryptogams; two were isolated from the green alga *Mougeotia scalaris* Hass. (Suetsugu *et al.*, 2005), and four from ferns (one full-length sequence from *Adiantum* and three short fragments from *Dryopteris filix-mas* (L.) Schott, *Onoclea sensibilis* L. and *Hypolepis punctata* Mett., respectively) (Kawai *et al.*, 2003). Thus, *NEO* sequence information is still extremely limited. Here we report the isolation and sequence analyses of the full-length genomic *NEO* sequences from two polypod ferns *Coniogramme intermedia Hieron.* var. *glabra* Ching, *Pronephrium lakhimpurnense* (Ros.) Holtt., and one tree fern *Plagiogyria distinctissima* Ching. Phylogenetic analyses were conducted to discern the gene origin, and the secondary and tertiary structures of deduced proteins were further predicted using bioinformatics methods.

Materials and Methods

Plant materials and DNA isolation.—All ferns were cultivated at Wuhan Botanical Garden, Chinese Academy of Sciences, Hubei, China. For each plant,

TABLE 1. Primers used in diversified genome-walking experiments.

Methods	Oligo name[a]	Sequence (5′→3′)
IPCR	5′ GDP F1	ATCACGCCCAATTCCGAY(A/C/T)TTC
	5′ GSP R1	CACAGCATTGTCTGCGTCATAAG
	5′ GSP F2	GTTCTGGTTCAGGTCGCATAC
	5′ GSP R2	AGAAGACCCCACCAGGCTAATG
Universal PCR	5′ GSP F3	TCAGCAATGTCCGCCAAGACGAAG
	5′ GSP R3	TAGGCCATCACCCGATCGTAGC
	GSP F1	GATGGCCTACAAGTTCCACGAG
	GSP R1	TGGCAGGGGTAGTGATAGA
	GDP F2	ATCACGCCCAATTCCGAY(A/C/T)TTC
	GDP R2	TGH(C/T)ACTCCGATGAAGTACTGGA
	GSP F3	GAGGTGCTCGGTGAGAACTG
	GSP R3	CTCGGGCTTGAGGTCTCGGTAG
SON	3′ GSP 1	TGAGGAATCTGCCCGCTTCTACGC
	3′ GSP 2	CCACTGCATGGGTGTCATCTAC
IPCR	3′ GSP F2	TCAAGCCCGAGAACCTGCTACTG
	3′ GSP R2	ATCCCTCCGTGTACTTGCTAACC
	3′ GSP F3	GAGGACTGGTGGGCGTTGGGAATC
	3′ GSP R3	CGGTAAGATTGAAGAACCCTGTG

[a] GSP referred to genomic specific primer, while GDP referred to degenerated primer.

three pieces of fresh leaves were collected to isolate genomic DNA following modified CTAB protocols (Sambrook *et al.*, 2001).

Cloning and sequencing of full-length NEO gene from C. intermedia var. *glabra.*—Full-length genomic *NEO* genes were amplified using diversified genome-walking PCR approaches. Primers used are listed in Table 1. Specific and degenerate primers were designed to amplify the internal *NEO* regions of *C. intermedia* var. *glabra* based on the conserved phytochrome chromophore-binding and phototropin regions of between *Adiantum NEO* (Accession No.: AB012082) and *M. scalaris* homologous sequences (Accession No.: AB206967, AB206966). Using the primer pairs GSP F1/R1, GDP F2/R2 and GSP F3/R3, universal PCR amplifications were performed in 20 μl of reaction mixture containing 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100, 2.0 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 1 U *Taq* DNA polymerase, 0.4 μM primers, 50 ng genomic DNA, and DNA-free water. PCR amplifications were carried out under the following conditions: the template was denatured at 94°C for 5 min followed by 35 cycles of amplification (94°C for 30 s, 62°C for 30 s and 72°C for 90 s) and a final elongation step at 72°C for 10 min.

Meanwhile, Inverse PCR (IPCR; Huang, 1997) was used to obtain the 5′-portion of the gene. The genomic DNA of *C. intermedia* var. *glabra.* was digested with ApoI restriction enzymes and ligated with T4 DNA ligase. Then the ligation solution was used as the PCR template. The first round and nested PCR amplification were performed with primer pairs 5′ GDP F1/5′ GSP R1, 5′ GSP F2/5′ GSP R2 and with LA *Taq*E following the procedures the same as described above except that the annealing temperature was 56°C.

The 3′ *NEO* sequence of *C. intermedia* var. *glabra* was cloned by single oligonucleotide nested PCR (SON PCR; Antal *et al.*, 2004) using primers 3′ GSP 1 and 3′ GSP 2 (Table 1) for the first and second round PCR, separately. The DNA template for the first round PCR was 50 ng of genomic DNA. The PCR program was: one denaturation step for 3 min at 94°C, five cycles of amplification (30 s at 94°C, 1 min at 60°C, 3 min at 72°C) followed by one ramping step (30 s at 94°C, 3 min at 29°C, 3 min ramp to 72°C, 2.5 min at 72°C) and 60 new amplification cycles (30 s at 94°C, 1 min at 60°C, 2.5 min at 72°C) with a 10-min extension step at 72°C. The second round PCR used 2 μl of a 1:50 dilution of the first round PCR product as the template, and was performed with the following conditions: 5-min denaturation at 94°C, 30 cycles of amplification (30 s at 94°C, 1 min at 60°C, 2.5 min at 72°C) and 10-min terminal extension at 72°C.

All PCR products amplified by the above steps were recovered from 1% agarose/TAE gels and purified with a quick PCR Purification Kit (Promega), and then cloned into PMD19-T vectors (Takara). The plasmids were transformed to *Escherichia coli* strain DH5α. Based on the color reaction using Xgal-IPTG system and PCR identification, white transformants were selected and positive clones were sequenced with an ABI PRISM 3730 DNA analyzer. Three clones were sequenced for each amplicon to control *Taq* polymerase errors. The overlapping sequences from various amplification steps were assembled in a single contig.

Isolation of NEO genes from P. distinctissima and P. lakhimpurnense.—The internal regions of *NEO* genes for *P. distinctissima* and *P. lakhimpurnense* were amplified by using the same primers and PCR procedures as *C. intermedia* var. *glabra*. Primers 5′ GSP F3 and 5′ GSP R3 were used to obtain the 5′ regions. The former was designed based on the 5′ conservative *NEO* sequences between *A. capillus-veneris* and *C. intermedia* var. *glabra*, while the latter according to the internal regions obtained above. The 3′ *NEO* region of *P. distinctissima* was obtained using the same SON PCR procedures and primers as those of *C. intermedia* var. *glabra*, whereas the 3′ sequence of *P. lakhimpurnense NEO* was gained by IPCR with ApoI restriction enzyme system and the primer pairs 3′ GSP F2/ R2 and 3′GSP F3/ R3. Amplified PCR products were purified, cloned, sequenced and assembled as described above.

Sequence accession numbers.—*NEO* gene sequences were submitted to NCBI database with the Accession Numbers FJ501964–FJ501966.

Sequence analysis and prediction of the NEO *structure.*—The open reading frame (ORF) finder and BLAST tool were used to predict coding sequence and estimate similarities of *NEO* with other genes. The translated amino acid sequences of each gene were analyzed using the program Protparam on Expasy website. Protein domain search, prediction of protein hydrophobicity, transmembrane structures and signal peptide cleavage sites were performed with Prosite (Hofmann *et al.*, 1999), ProtScale (Gasteiger *et al.*, 2005), TMpred (Hofmann and Stoffel, 1993) and SignalP (Emanuelsson *et al.*, 2007) programs, respectively. SOPMA (Geourjon and Deleage, 1995) analyses were carried out to predict the secondary structure. Three-dimensional (3D) structures of

functional domains were computer-modeled by SWISS-MODEL (http://www.isb-sib.ch/) program. 3D structures of PAS (Per / Arnt / Sim) and GAF (cGMP specific phosphodiesterase / adenylate cyclases / formate hydrogen lyase transcription) were predicted based on the 1.45 Å crystal structure of *Deinococcus radiodurans* Phytochrome (PDB code: 2o9c). The LOV (Light, Oxygen and Voltage-sensing) 1, LOV2 and STKc (Serine/Threonine protein kinases, catalytic) domains were computer-modeled using fungal photoreceptor VVD (PDB code: 3d72A; Resolution: 1.65 Å), *Adiantum* NEO LOV2 (PDB code: 1jnuB; 2.60 Å) and Human Myotonic Dystrophy Protein Kinase (PDB code: 2vd5A; 2.80 Å) as templates, respectively. Models were displayed with the PyMol program (DeLano, 2002).

Sequence alignment and phylogenetic analysis.—Other than the sequences cloned in this work, sequences used for comparison and phylogenetic analysis were downloaded from Genbank. Complete species names and accession numbers are listed in Table 2. Based on the predicted structure of NEO, we divided the *NEO* sequences data into N-terminal phytochrome-like and C-terminal phototropin-like regions. Sequences were aligned using Clustal X 2.0 and then adjusted manually. The phylogenetic trees were constructed from the aligned coding DNA using the maximum likelihood (ML) method as implemented in PHYML (Guindon and Gascuel, 2003). Before ML analyses, we first identified the best fit model of molecular evolution for the N- and C-terminal datasets using the Akaike Information Criterion (AIC) as implemented in ModelTest (Posada, 2003). The model of the general time reversible (GTR) + 4G (four categories of Gamma substitution rates) + I (invariable sites) model were applied to each datasets. A nonparametric bootstrap test was conducted with 1000 replicates.

Results and Discussion

Cloning and sequence analysis of the complete coding sequences.—Using diversified genome-walking PCR approaches and the primers in Table 1, the *NEO* fragments were amplified from three fern species (Fig. 1). After cloning and sequencing, the assembling results displayed that the *NEO* genes from three fern species, *C. intermedia* var. *glabra*, *P. distinctissima*, and *P. lakhimpurnense*, are 4937 bp, 4424 bp, and 4456 bp in length, respectively. Comparative analyses with the full-length *A. capillus-veneris* L. *NEO* (*AcNEO*) cDNA sequence showed that introns are all absent in these genes. Ahead of the original codon ATG and after the stop codon TGA, there are 294-bp, 6-bp and 6-bp 5′- untranslated regions (UTR), in addition to a 311-bp, 100-bp and 139-bp 3′-UTR for *C. intermedia* var. *glabra*, *P. distinctissima*, and *P. lakhimpurnense*, respectively. Each sequence consists of an ORF of 4332 (*C. intermedia* var. *glabra*), 4308 (*P. distinctissima*) and 4317 bp (*P. lakhimpurnense*), which is very similar to that of *AcNEO* (4398 bp). Their guanine–cytosine (GC) contents are 59.5%, 61.0% and 58.7%, respectively. The three genes each encode a deduced protein of 1443, 1435 and 1438 amino acid residues with a

TABLE 2. Species names and accession numbers of nucleotide acid sequences used for comparison and phylogenetic analysis.

Species	Gene names	Accession numbers	Species	Gene names	Accession numbers
Adiantum capillus-veneris	AcPhy1	AB016168	*Pinus sylvestris*	PsPhyP	EU203168
Adiantum capillus-veneris	AcPhy2	AB016232	*Sorghum bicolor*	SbPhyB	AY466087
Arabidopsis thaliana	AtPhyA	X17341	*Selaginella martensii*	SmPhy1	X61458
Arabidopsis thaliana	AtPhyB	X17342	*Solanum tuberosum*	StPhyB	Y14572
Arabidopsis thaliana	AtPhyC	X17343	*Zea mays*	ZmPhyB1	AY234827
Arabidopsis thaliana	AtPhyD	X76609	*Zea mays*	ZmPhyB2	AY234828
Arabidopsis thaliana	AtPhyE	X76610	*Adiantum capillus-veneris*	AcPhot1	AB037188
Ceratodon purpureus	CpPhy1	U87632	*Adiantum capillus-veneris*	AcPhot2	AB115545
Ceratodon purpureus	CpPhy2	U72993	*Avena sativa*	AsNph1-1	AF033096
Ceratodon purpureus	CpPhy3	AY123149	*Avena sativa*	AsNph1-2	AF033097
Ceratodon purpureus	CpPhy4	EU122393	*Arabidopsis thaliana*	AtPhot1	NM_114447
Pomoea nil	InPhyE	U39787	*Arabidopsis thaliana*	AtPhot2	NM_180879
Lycopersicon esculentum	LePhyB1	AJ002281	*Chlamydomonas reinhardtii*	CrPhot	AJ416557
Lycopersicon esculentum	LePhyF	U32444	*Lycopersicon esculentum*	LePhot1	EF063359
Lotus japonicus	LjPhyB	AB264087	*Lycopersicon esculentum*	LePhot2	DQ886531
Mesotaenium caldariorum	McPhy	U31284	*Mougeotia scalaris*	MsPhotA	AB206963
Marchantia paleacea	MpPhy	AB022917	*Mougeotia scalaris*	MsPhotB	AB206964
Mougeotia scalaris	MsPhy1	AB206965	*Oryza sativa*	OsNph1	AJ252142
Mougeotia scalaris	MsNeo1	AB206966	*Oryza sativa*	OsNph1a	AB018444
Mougeotia scalaris	MsNeo2	AB206967	*Oryza sativa*	OsNph1b	AB018443
Nicotiana plumbaginifolia	NpPhyB	Y14676	*Physcomitrella patens*	PpPhotA1	XM_001774204
Neurospora crassa	NcPhy1	DQ128077	*Physcomitrella patens*	PpPhotA2	XM_001774562
Oryza sativa	OsPhyA	AB109891	*Physcomitrella patens*	PpPhotA3	XM_001765356
Oryza sativa	OsPhyB	AB183525	*Physcomitrella patens*	PpPhotA4	XM_001763052
Oryza sativa	OsPhyC	AF141942	*Physcomitrella patens*	PpPhotB1	XM_001766357
Picea abies	PaPhyO	U60264	*Physcomitrella patens*	PpPhotB2	XM_001785674
Populus balsamifera	PbPhyB1	AF309806	*Physcomitrella patens*	PpPhotB3	XM_001755269
Populus balsamifera	PbPhyB2	AF309807	*Pisum sativum*	PsPhot1	AY093427
Physcomitrella patens	PpPhy1	XM_001778103	*Phaseolus vulgaris*	PvPhot1a	AB204872
Physcomitrella patens	PpPhy2	XM_001782287	*Phaseolus vulgaris*	PvPhot1b	AB204873
Physcomitrella patens	PpPhy3	AB275306	*Phaseolus vulgaris*	PvPhot2	AB204874
Physcomitrella patens	PpPHY4	XM_001773498	*Vicia faba*	VfPhot1a	AB095909
Physcomitrella patens	PpPHY5a	XM_001761093	*Vicia faba*	VfPhot1b	AB095910
Physcomitrella patens	PpPHY5b3	XM_001767172	*Zea mays*	ZmPhot1	NM_001111416
Physcomitrella patens	PpPhy5c	XM_001754314			

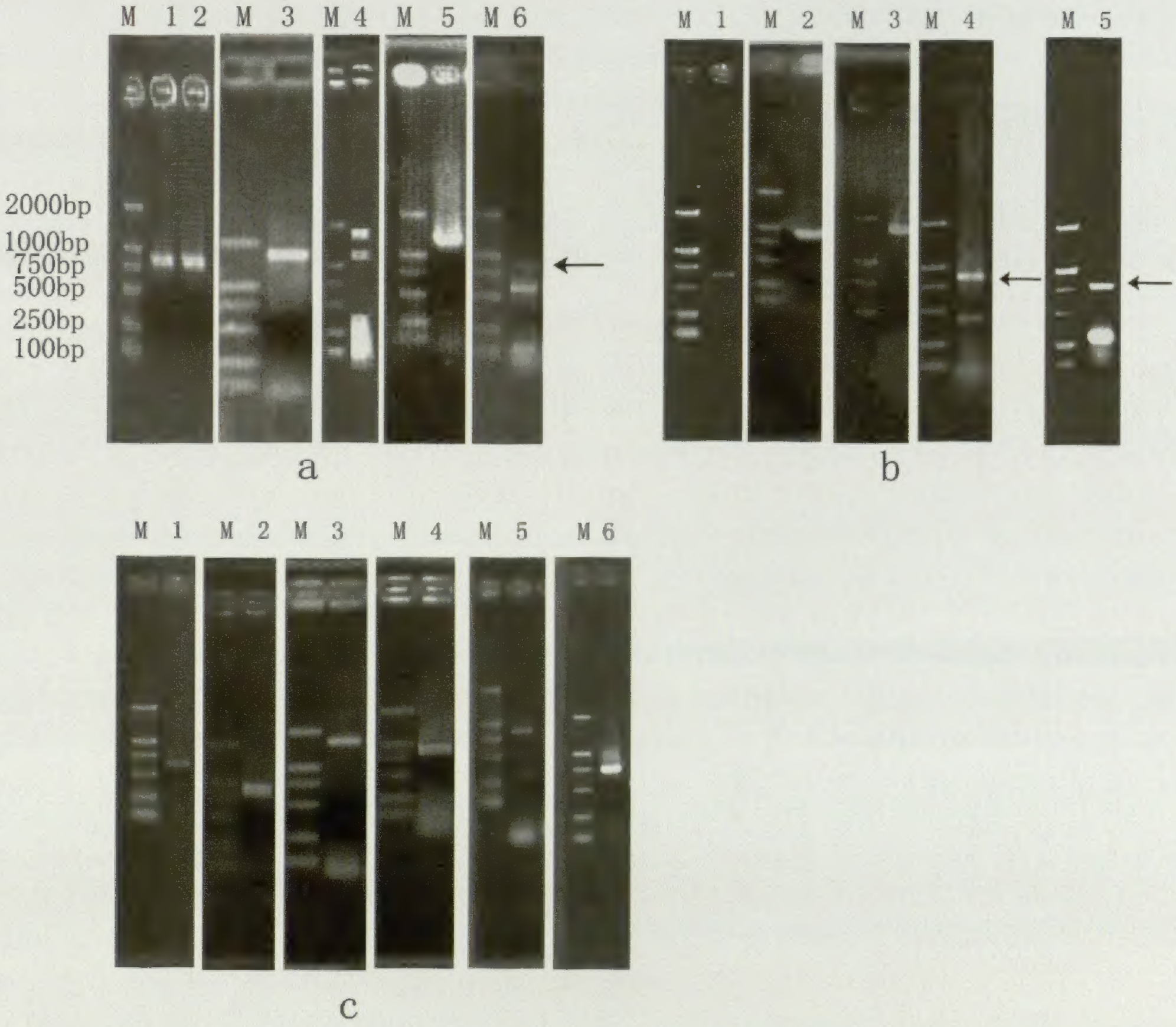

FIG. 1. Agarose gel electrophoresis of amplification products for *Coniogramme intermedia*. var. *glabra* **(a)**, *Plagiogyria distinctissima* **(b)**, and *Pronephrium lakhimpurnense NEO* fragments **(c)**. M is molecular weight marker (DL2000). **a1** and **a2:** the products amplified by the primer pairs GSP F1/R1and GSP F3/R3; **a3:** the products amplified by the primer pairs GDP F2/R2; **a4** and **a5:** the products amplified by the first and second IPCR using the primer pairs 5′ GDP F1/5′ GSP R1 and 5′ GSP F2/5′ GSP R2, respectively; a6: the amplification products of SON PCR by the primer 3′ GSP 2. **b1:** the products amplified by the primer pairs 5′ GSP F3/5′ GSP R3; **b2:** the products amplified by the primer pairs GSP F1/R1; **b3:** the products amplified by the primer pairs GDP F2/R2; **b4:** the products amplified by the primer pairs GSP F3/R3; **b5:** the amplification products of SON PCR by the primer 3′ GSP 2. **c1:** the products amplified by the primer pairs 5′ GSP F3/5′ GSP R3; **c2:** the products amplified by the primer pairs GSP F1/R1; **c3:** the products amplified by the primer pairs GDP F2/R2; **c4:** the products amplified by the primer pairs GSP F3/R3; **c5** and **c6:** the amplification products of the first and second IPCR by the primer pairs 3′ GSP F2/ R2 and 3′GSP F3/ R3, respectively.

theoretical isoelectric point (pI) of 6.44, 6.27 and 6.28 and a calculated molecular mass about 160.15, 158.51 and 158.28kDa.

Amino acid compositions of the three deduced proteins are very similar to each other. The richest amino acid is Leu (9.8~10.5% by frequency), followed by Ala (8.0~8.7%), Gly (7.8~8.7%), Ser (7.3~7.8%) and Arg (7.1~7.5%). Acidic and basic amino acids constitute about 12% and 11% of the protein separately. Of the total amino acids, 23% are polar while 32% charged. The

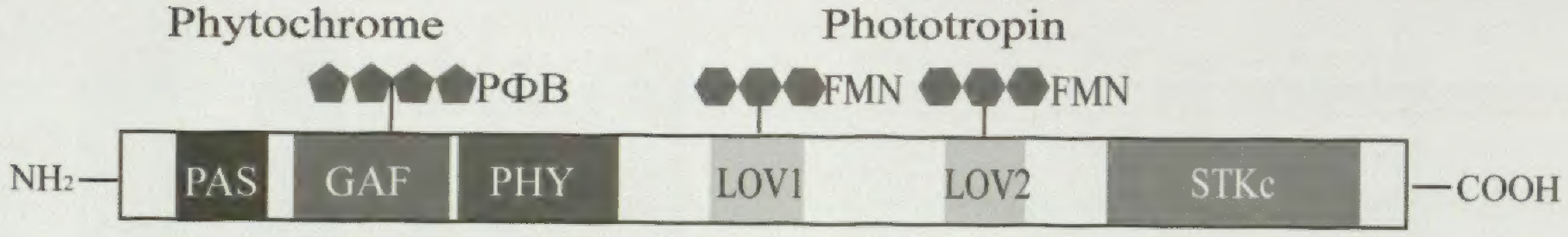

FIG. 2. The common structure of NEO in fern species. It shows that the N-terminal phytochrome chromophore domain contains PAS, GAF, and PHY domains and C-terminal phototropin motif possesses two LOV and one STKc domains.

deduced NEO proteins were predicted to have a net charge of −5.1, −9.2, and −7.9 at pH 7.0. They each contain 488, 495 and 494 hydrophobic amino acids (33.8%, 34.5% and 34.4% by frequency), and are all predicted to have a number of typical hydrophobic regions identified by Kyte and Doolittle hydropathy analyses (Gasteiger *et al.*, 2005). Each protein possesses several helices thought to be transmembrane domains with the N- terminal domains on the intracellular side. However, analysis of the primary structure did not reveal any signal peptide, indicating that they are most probably cytoplasmic. The results are in accordance with *Ac*NEO, which is a plasma-membrane localized protein (Nozue *et al.*, 1998). However, its putative association with the membrane remains unclear.

The three genes show 78~80% identity with *AcNEO* at the nucleotide acid level as well as >88% identity among themselves (Table 3). The N-terminal sections of *NEO* exhibits 40~58% identity with seeded vascular and non-vascular plant *PHY*s, and the C-terminal regions show 40~52% identity with plant *PHOT*s. Protein domain searches displayed that all three proteins possess the typical features of fern NEO family. They share three domains of phytochrome photosensory core region in the N-terminus and a nearly full phototropin homolog in the C-terminus (Fig. 2). Henceforth, we denoted the three new fern *PHY-PHOT* chimera genes as *C. intermedia* var. *glabra NEO* (*CiNEO*), *P. distinctissima NEO* (*PdNEO*), and *P. lakhimpurnense NEO* (*PlNEO*), respectively.

By contrast, the sequences of the chimeric photoreceptors are not conserved between the fern and algae. Fern *NEO*s are all distantly related to *Mougeotia scalaris NEO* (*MsNEO*)1 and *MsNEO*2 with <27% identity (Table 3). *MsNEO*1 and *MsNEO*2 exhibit lower identity to seeded vascular and non-vascular plant *PHY*s (<46%) and *PHOT*s (<45%) compared with fern *NEO*. In combination with the results that fern *NEO* genes are intronless but *MsNEO*1 and *MsNEO*2 have many introns (Nozue *et al.*, 1998; Suetsugu *et al.*, 2005), we also speculate that fern and alga *NEO* arose independently and evolved convergently. This is in agreement with a previous study (Suetsugu *et al.*, 2005). However, it cannot be excluded that the fern *NEO*s might have experienced intron losses during their evolution.

Phylogenetic analysis.—Fern and alga *NEO*s each constitute a clade with strong statistical support in the ML trees (Fig. 3). They further form a major clade in the tree established from the sequences of phytochrome sensor domains, while seeded vascular and non-vascular plants (including the

TABLE 3. Percentage of sequence identity among fern and alga *NEO*, canonical phytochromes, and phototropins.[a]

	AcNEO	*CiNEO*	*PdNEO*	*PlNEO*	*MsNEO1*	*MsNEO2*
AcNEO						
CiNEO	78.6					
PdNEO	79.5	89.4				
PlNEO	78.3	88.0	89.1			
MsNEO1	26.3	26.5	26.6	26.3		
MsNEO2	26.8	26.8	27.1	26.8	55.9	
Plant *PHY*[b]	41.2~56.8	41.1~57.2	41.2~57.6	40.7~56.1	40.6~45.5	39.9~45.5
Plant *PHOT*[b]	40.5~51.7	40.3~51.3	40.3~51.9	40.6~51.3	33.2~45.1	33.3~45.0

[a] The sequences of *CiNEO*, *PdNEO* and *PlNEO* were obtained by cloning and sequencing in this work, and the others were downloaded from Genbank database. Complete species names and accession numbers were listed in TABLE 2.

[b] The alignment with plant *PHY* and *PHOT* used the phytochrome-like N-terminal and phototropin-like C-terminal region, separately. Plant *PHY* and plant *PHOT* were in accordance with *PHY* and *PHOT* in molecular evolution analysis below.

angiosperm, gymnosperm, fern, moss, liverwort and alga) comprise another (Fig 3a). By contrast, for the ML tree constructed using the sequences of phototropin domains (Fig 3b), the fern *NEO*s were grouped together with seeded vascular and non-vascular plant *PHOT*s rather than alga *NEO*s. However, in each tree none of these clades received strong support. *NEO* has been regarded as the fusion between phytochrome and phototropin motifs (Suetsugu *et al.*, 2005). Since a robust phylogeny cannot be reconstructed here, we must await more necessary data to resolve the origin of fern *NEO* genes.

Predictions of secondary and tertiary structure.—The predicted secondary structures exhibit that the putative NEO polypeptides all contain about 37% alpha helices, 17% extended strands, 9% β turns and 37% random coils. Alpha helices and random coils constitute the interlaced domain of the main part of the secondary structure.

The predicted 3D structure of functional domains in *Ci*NEO, *Pd*NEO, and *Pl*NEO are displayed in Fig. 4. The 3D structure models of PAS and GAF domains were constructed with E-values of $2.9E^{-28}$, $1.8E^{-30}$, and $1.2E^{-29}$ for *Ci*NEO, *Pd*NEO, and *Pl*NEO, respectively. The PAS and GAF domains of the three proteins are highly conserved, which all share the characteristic structure of plant phytochromes (Wagner *et al.*, 2005). The main difference among *Ci*NEO, *Pd*NEO, and *Pl*NEO is that *Pd*NEO has a longer β-sheet at GAF domain, but *Ci*NEO and *Pl*NEO are random coils in that position. However, the principle of this difference remains unclear. Except for that, the three proteins each contain a figure-eight knot donated by GAF and PAS domains, and they all reserve the conserved cysteine residue (Cys306 for *Ci*NEO, Cys300 for *Pd*NEO and Cys301 for *Pl*NEO) in the GAF domain, which is associated with phytochromobilin (PΦB) by a covalent linkage (Boylan and Quail, 1991). The adjacent histidine residue (His307 for *Ci*NEO, His301 for *Pd*NEO and His302 for *Pl*NEO), required for tight chromophore-protein interaction, is also conserved.

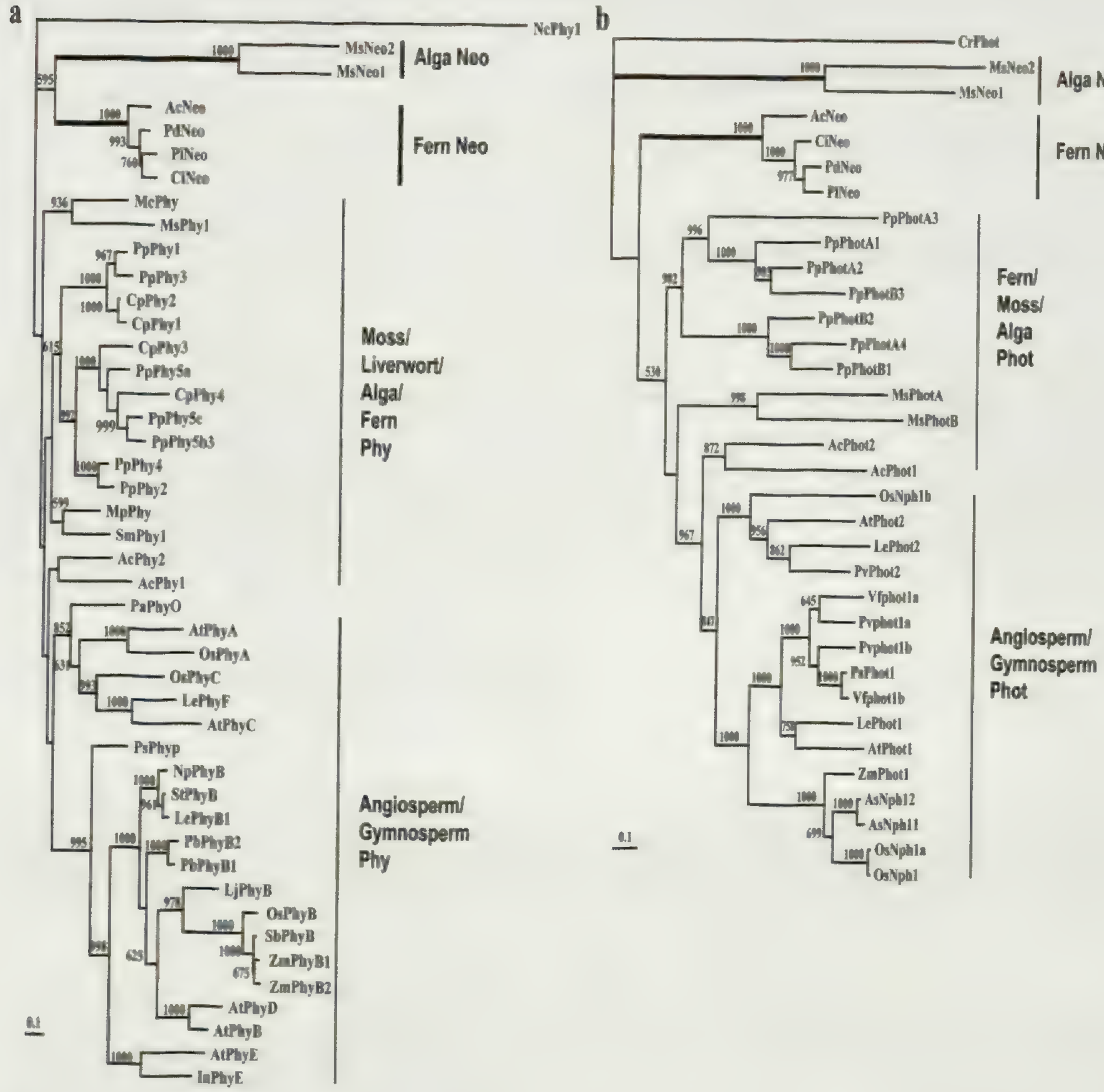

FIG. 3. Phylogenetic trees established from nucleotide acid sequences of phytochrome sensor domains **(a)** and phototropin domains **(b)** generated by the maximum likelihood method using PHYML software, which are arbitrarily rooted with *Neurospora crassa* phytochrome (NcPhy1) and *Chlamydomonas reinhardtii* blue light receptor (CrPhot). Numbers above branches or near to the right of nodes indicate bootstrap values (only values>500 are indicated) obtained from 1000 replications. The scale bar represents substitution number per nucleotide site. Neo branches are marked with bold dark lines.

The LOV1 and LOV2 structures of three NEO are nearly identical (Fig. 4b and 4c), which is consistent with the high degree of sequence conservation at the amino acid level (Fig. 5). The LOV domains all possess the typical α/β fold of the PAS family, comprising five stranded anti-parallel β-sheets (β1–β5) and three α-helical segments (α1–α3), which are with a backbone β-scaffold and a helical connector forming a tight cage enclosing the FMN (Pandini and Bonati, 2005). All LOV domains contain the highly conserved motif NCRFLQ and constitute a single turn of a 3_{10} helix (a′A), contributing to the chemistry of photoactivation and subsequent downstream signaling (Crosson and Moffat, 2001). The LOV domains also contain the conserved flavin-binding residues

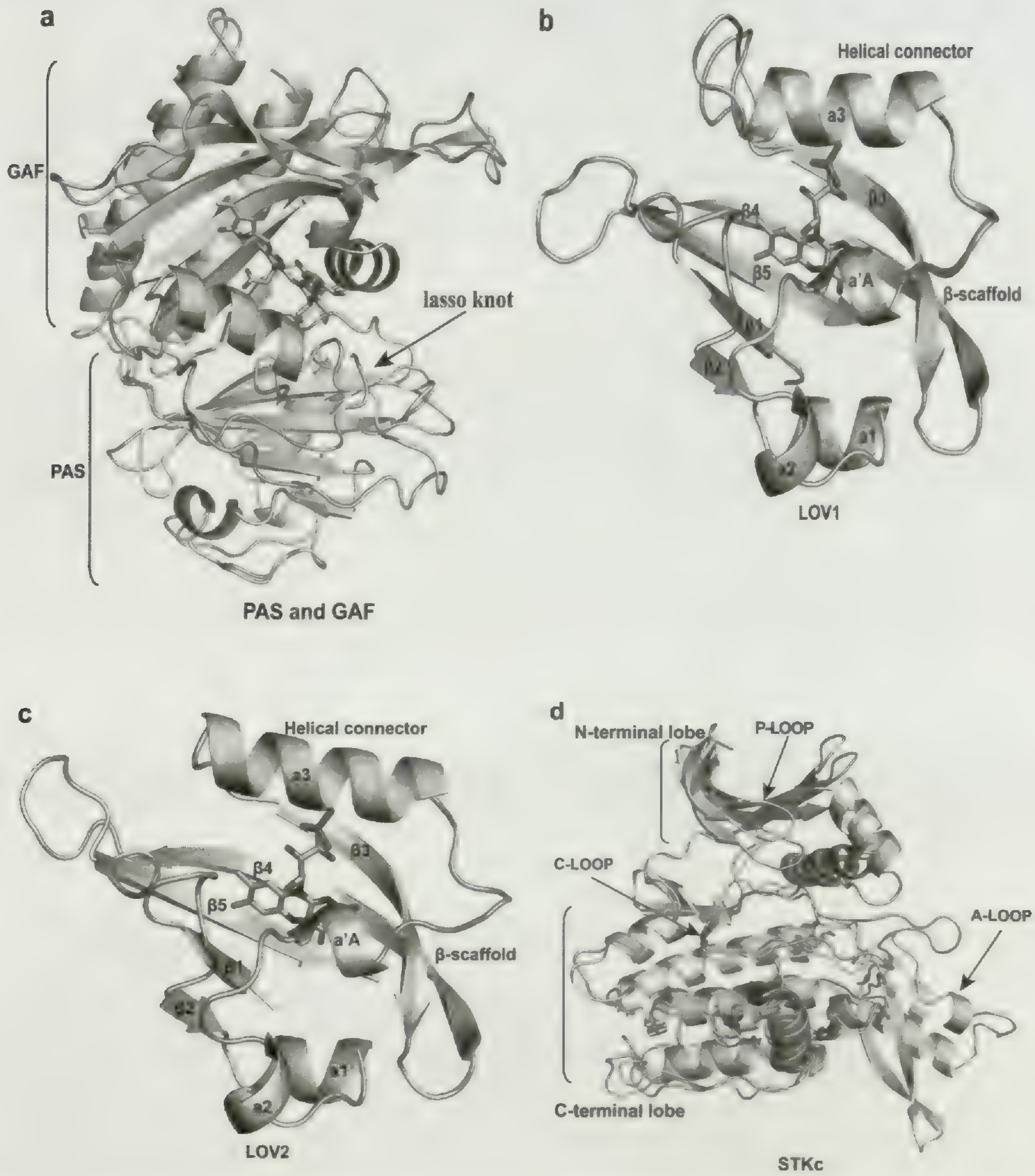

FIG. 4. The alignment of the predicted three-dimensional structure of NEO functional domains for three fern species *Coniogramme intermedia* var. *glabra* (dark gray), *Plagiogyria distinctissima* (gray) and *Pronephrium lakhimpurnense* (light gray). **(a)** The predicted 3D structure of PAS and GAF domains. The phytochromobilin (PΦB) linked with cys residues are displayed with sticks. The figure-eight knots are marked with black arrow. **(b)** and **(c)** The predicted 3D structure of LOV1 and LOV2 domains, respectively. The FMN ligand linked with cys residues are displayed with sticks. **(d)** The predicted 3D structure of STKc domains. Areas of the P-loop (phosphate-binding loop), C-loop (catalytic loop) and A-loop (activation loop) are marked with three black arrows.

including the photochemically active cysteine residues (Cys712, 982 for *Ci*NEO; Cys702, 973 for *Pd*NEO; and Cys707, 977 for *Pl*NEO). Furthermore, 11 residues that interact with FMN were identified based on the results of the flavin-binding domain (Crosson and Moffat, 2001). Residues in a′A and a3

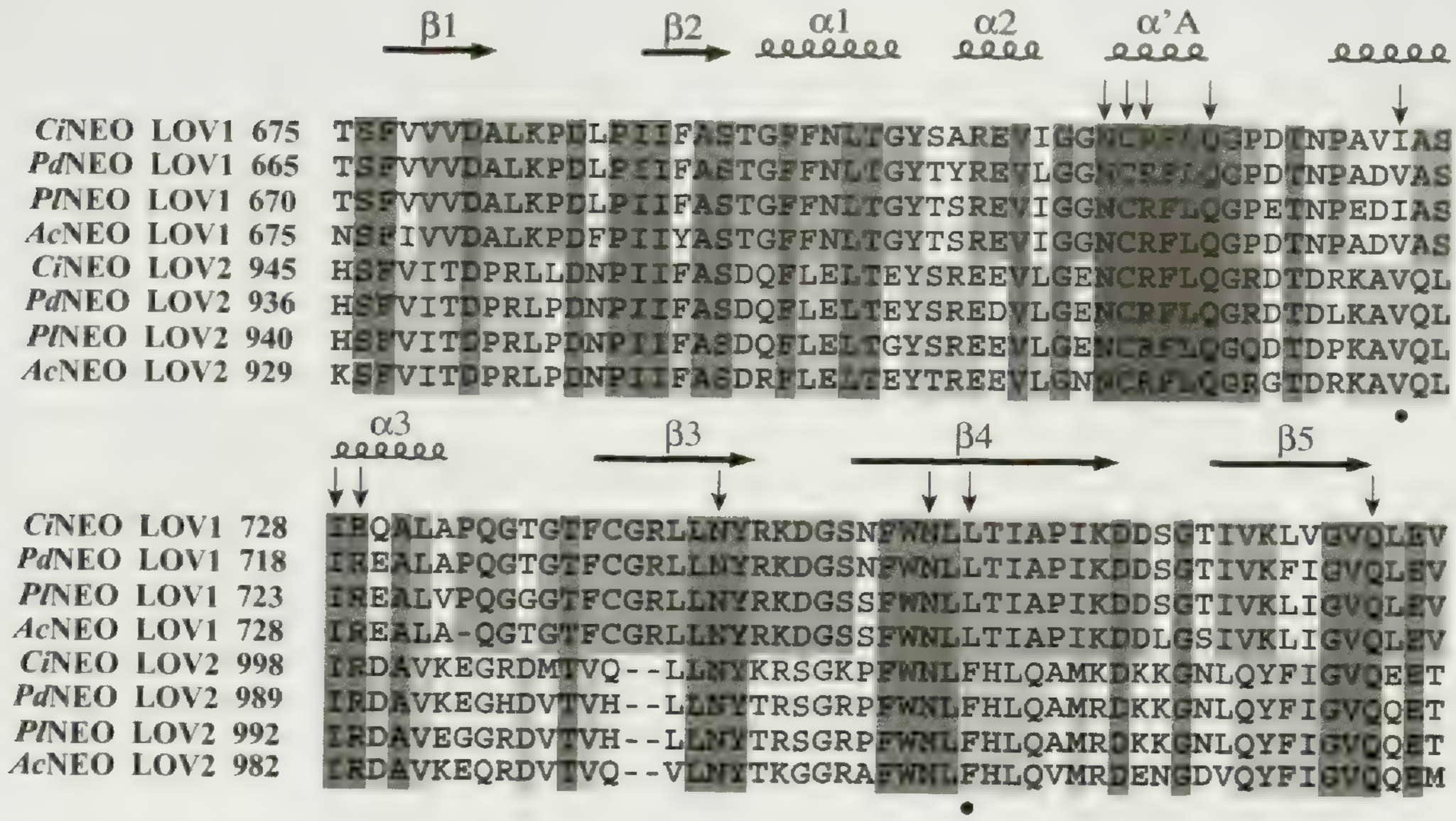

FIG. 5. Alignment of the eight LOV domains and the identification of flavin-interacting residues of *Coniogramme intermedia* var. *glabra*, *Plagiogyria distinctissima* and *Pronephrium lakhimpurnense*. NEO proteins. Secondary structure of LOV is marked above alignment: β-strand, arrows; helix, windings. Dark gray background residues are conserved in all LOV domains. Gray residues are conserved in all LOV1 domains, whereas light gray residues are conserved in all LOV2 domains. NCRFLQ residues are specific motif in LOV domains. Residues that interact with FMN are marked above with black vertical arrows, whereas variable residues interacting with FMN marked bottom with black dots.

helixes as well as β-strands 3 and 4 make the majority of flavin contacts. Site-directed mutagenesis analysis suggested that the FMN chromophore interacting site Gln1029 in *Adiantum* (corresponding to Gln1045 in the LOV2 of *Ci*NEO, Gln1036 in *Pd*NEO and Gln1039 in *Pl*NEO) plays an important role in the protein structural changes of NEO-LOV2. Most of the FMN interacting residues are conserved in the aligned LOV domains with only two exceptions. One is the residue 725 (*Ci*NEO LOV1 as reference), which is an isoleucine in *Ci*NEO and *Pl*NEO LOV1 but a valine in other LOV1 and all LOV2; the other is the residue 758, which is a leucine in LOV1 but a phenylalanine in LOV2 (Fig. 5). For *Adiantum* NEO, it has been suggested that the two amino acid differences between LOV1 and LOV2 is relative to the distinct roles that they play in the photoregulation process (Yamamoto *et al.*, 2008).

The NEO kinases of three fern species all have a α/β structure similar to PKA (c-AMP-dependent protein kinase A) containing the autophosphorylation site (Akamine *et al.*, 2003) as shown in Fig. 4d. Similarly, the NEO STKc domains all fold into a two-lobed structure including one smaller N-terminal and one larger C-terminal lobes. Based on the information of functional sites in PKA (Shaltiel *et al.*, 1998), we examined the key sites in fern NEO. The N-terminal lobe in the STKc domains contains the glycine rich and conserved motif GSGDTG as well, which contribute to the P-loop (phosphate-binding loop) of

PKA, acting as an ATP-binding site (Akamine *et al.*, 2003). The C-terminal lobe contains the typical amino acid sequences of the C-(catalytic) and the A-(activation) loop of PKA. In the C-loop regions, all the residues are conserved including the phosphorylation reaction catalyzing residue (Asp1253, Asp1244, and Asp1247 for *Ci*NEO, *Pd*NEO, and *Pl*NEO, respectively). For the A-loop, *Ci*NEO is formed mainly by two β-sheets, *Pd*NEO by two α-helixes while *Pl*NEO by one α-helixe and two small β-sheets. Whether this difference contributes to the activation ability remains unknown. By contrast, the Asp residue responsible for chelating Mg^{2+} ions and the consensus phosphorylation site (Ser1313, Ser1305 and Ser1308 for *Ci*NEO, *Pd*NEO, and *Pl*NEO, respectively), which have been reported to be required for full kinase activity (Tokutomi *et al.*, 2008), are conserved in all the NEO STKc domains. In summary, the NEO STKc domains for three fern species well preserve the PKA functional motifs, suggesting that NEO may have a molecular mechanism similar to PKA.

Conclusion

In this study, we have described the isolation and sequence analyses of full-length *NEO* genes from one tree fern and two polypod ferns. This may suggest that *NEO* genes are widely exist in many fern species (at least in tree ferns and polypod ferns). Molecular characterization of the three *NEO* genes reveals that their ORF size, GC content, basic protein properties and the predicted secondary and 3D structures are very similar to those of *Adiantum NEO*. In fact, the major structural motifs are all identical in fern NEOs, implying that the NEO may share a common mechanism for light-sensing in different fern species. The minor differences among them may relate to the individual diversification. Its N-terminus has three domains with homology to phytochrome and C-terminus contains three domains similar to phototropin. The comparisons of the PAS and GAF structures with plant phytochromes and STKc domain with PKA also reveal that the NEO well preserves the functional motifs. The analysis of the functional domains and key sites relating to both regulation and catalyzation may provide useful information on the molecular mechanism underlying the photoregulation mediated by NEO proteins.

Acknowledgments

This project was supported by the "100 Talent Project" of Chinese Academy of Sciences (Grant No.: 0729281F02), the National Natural Science Foundation of China (Grant No.: 30771763, 30170101), and the "Outstanding Young Scientist Project" of the Natural Science Foundation of Hubei Province, China (Grant No.: 0631061H01).

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American Fern Journal 100(1):16–26 (2010)

Mycorrhizal Status of Obligate and Facultative Epiphytic Ferns in a Valdivian Temperate Forest of Patagonia, Argentina

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ABSTRACT.—Arbuscular mycorrhizae (AM) influence the growth, morphology and fitness of the plant species they colonize. Despite the abundance and importance of epiphytes in forest dynamics, little is known about AM in these plants. Abundant epiphytes are present in the Valdivian temperate forests of South America, where ferns are one of the most important components of the epiphytic vascular flora. The aim of this work was to analyze the occurrence of AM in sporophytes of obligate and facultative epiphytic fern species in a Valdivian temperate forest of Patagonia, Argentina. We examined the roots of 10 epiphytic fern species. Roots of 66 sporophytes were collected from tree trunks and branches between 0.5 and 2 m height above the ground and were stained by using a modified Phillips and Hayman (1970) method. Of the 10 species examined in the present study two were found to be obligate epiphytes, six were facultative epiphytes and two could not be classified because few individuals were found. Most of them lacked AM structures, except for seven *Hymenophyllum* sporophytes which were rooted in soil and possessed scarce knobby hyphae and structures similar to coils and vesicles. Abundant dark septate fungi were observed within the cortex of all samples. The absence of AM in epiphytes may be explained by different ecological or taxonomic reasons, but in this study we suggest that root morphology may be an important factor influencing mycorrhizal behavior in ferns.

KEY WORDS.—epiphytes, ferns, arbuscular mycorrhizae (AM), dark septate fungi (DSF), Patagonia

Autotrophic epiphytes are plants that grow upon other plants which only provide physical support and are not parasitized. Plants restricted to epiphytic habitats are called "obligate epiphytes" and those that occur both epiphytically and terrestrially are known as "facultative epiphytes" (Benzing, 1990; Janos, 1993). Vascular epiphytes constitute approximately 10% of all vascular flora (Kress, 1986) and play an important role in forest dynamics by contributing to species richness and by trapping and storing atmospheric nutrients, making them temporally unavailable to terrestrial plants (Benzing, 1990).

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Mycorrhizae generally influence plant fitness by improving nutrient uptake and increasing plant resistance to drought and other environmental stress factors (Smith and Read, 1997; Read, 1999). Despite the importance and abundance of epiphytes, little is known about mycorrhizal associations in these plants (Bermudes and Benzing, 1989; Allen *et al.*, 1993; Janos, 1993; Michelsen, 1993). Epiphytic habitats are usually considered extreme environments for plants because they often are subjected to great variation in temperature and in water and nutrient availability (Benzing, 1990). Therefore, it is generally expected that mycorrhizae may be an important adaptation for epiphytes (Benzing, 1990; Lesica and Antibus, 1990; Janos, 1993; Rains *et al.*, 2003). Nevertheless, it has been observed that epiphytic species are usually non-mycorrhizal (Allen *et al.*, 1993), facultative mycorrhizal (commonly mycorrhizal when they grow terrestrially and inconsistently mycorrhizal when they grow epiphytically) (Maffia *et al.*, 1993; Michelsen, 1993; Nadarajah and Nawawi, 1993), and that when they have AM, colonization is usually low (Bermudes and Benzing, 1989; Lesica and Antibus, 1990; Rowe and Pringle, 2005).

Studies concerning the occurrence of mycorrhizae in epiphytes are few and only the Orchidaceae and Ericaceae families have been relatively well studied (Bermudes and Benzing, 1989; Lesica and Antibus, 1990; Dearnaley, 2007). Some species included in the Bromeliaceae (Allen *et al.*, 1993; Rabatin *et al.*, 1993; Rowe and Pringle, 2005) and other predominant families of vascular epiphytes (such as the Araceae, Cyclanthaceae and Piperaceae) have also been analyzed (Bermudes and Benzing, 1989; Lesica and Antibus, 1990; Janos, 1993; Maffia *et al.*, 1993; Michelsen, 1993). However, the mycorrhizal status of epiphytic species included in families other than those mentioned here remains an open question. Therefore, much more information is needed before generalizations about mycorrhizae in epiphytic environments can be made (Bermudes and Benzing, 1989; Allen *et al.*, 1993; Janos, 1993).

Abundant epiphytes are present among the vegetation of the Valdivian temperate forests of South America. Within these forests, seedless vascular plants, mostly ferns (monilophytes, see Pryer *et al.*, 2004 and Smith *et al.*, 2006), are one of the most important components of the epiphytic vascular flora. The mycorrhizal status of this group of plants is poorly understood (Dhillion, 1993; Zhao, 2000) and much less is known about this symbiosis in epiphytic species.

This research was conducted in Puerto Blest, where previous studies have demonstrated the presence of AM in different plant species (Chaia *et al.*, 2006), including *Equisetum bogotense* HBK and *Lycopodium paniculatum* Desv. ex Poir., two species of seedless vascular plants which were found to be facultative mycorrhizal (Fernández *et al.*, 2008). The aim of the present study was to analyze the occurrence of AM in sporophytes of different obligate and facultative epiphytic fern species.

Materials and Methods

Study area.—The Valdivian temperate forests of South America are included in the Subantarctic Province (Subantarctic Domain, Antarctic Region). This

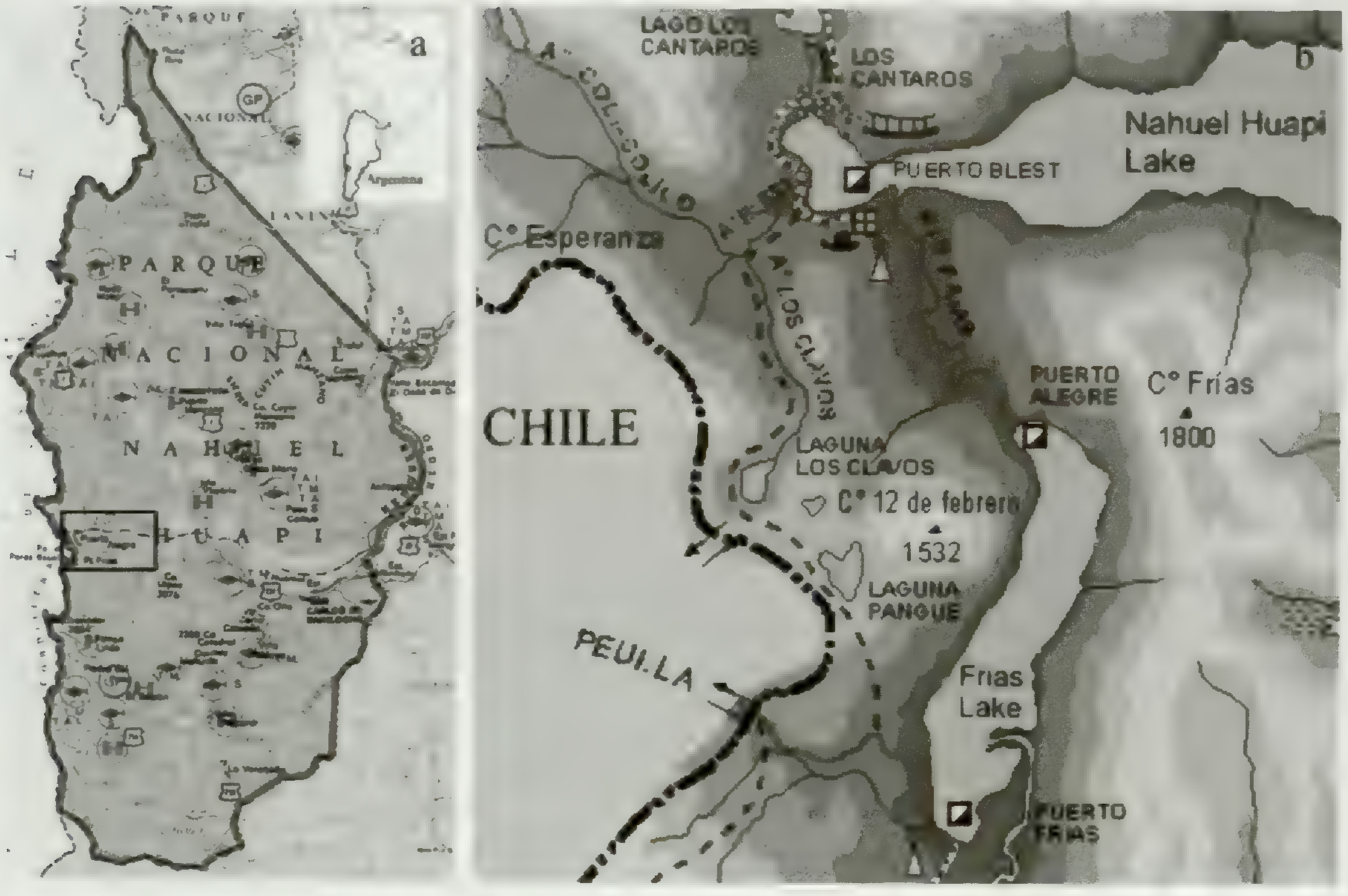

FIG. 1. Geographical location of the study area. **a:** Nahuel Huapi National Park, Puerto Blest area is pointed out in a black rectangle, **b:** Puerto Blest region.

ecoregion is located in the Southern region of Argentina and Chile, from 35° to 48° S latitude between the Eastern slope of the Andes and the Pacific Ocean. Puerto Blest (41° 02′ S, 71° 49′ W) is part of the Valdivian temperate forest region of Argentina and it is situated within the Nahuel Huapi National Park in Río Negro Province, Patagonia (Cabrera, 1976; Armesto *et al.*, 1995) (Fig. 1). It is one of the rainiest places in the country, with an average annual rainfall of 3000 mm and an annual average temperature of 9° C. The dominant tree species in this hydrophilic forest is *Nothofagus dombeyi* (Mirb.) Oerst. In some areas, this species forms mixed forests with *Fitzroya cupressoides* (Molina) IM Johnst., *Saxegothaea conspicua* Lindl., *Dasyphyllum diacanthoides* (Less.) Cabrera. and *Laureliopsis philippiana* (Looser) Schodde (Dimitri, 1972; Brion *et al.*, 1988; Correa, 1998). Trunks and branches of these tree species are usually covered by different epiphytes, including lichens, mosses and several ferns. Epiphytic ferns present in Puerto Blest are small plants that range in size from 2 to 20 cm.

Sampling.—Five samplings were conducted in Puerto Blest between spring 2004 and autumn 2006. Sporophytes of epiphytic ferns were sampled by random walk from trunks and branches of different tree species, such as *N. dombeyi*, *S. conspicua*, *D. diacanthoides* and *L. philippiana*, which were situated at least 5 m away from the footpath. The epiphytic specimens were collected from 0.5 to 2 m height above the ground. Species that were found to be also growing on the forest soil were considered as facultative epiphytes and root samples were collected from epiphytic as well as from the conspecific

terrestrial specimens. One individual of *Hymenophyllum dentatum*, a species that is reported to be epiphytic (Rodríguez, 1995) but was found only on the ground, was also sampled.

Sporophytes were carefully removed from the substrate in order to preserve most of the root system and were stored in labeled plastic bags and refrigerated at 4°C until processed. Roots from all samples were separated from the rest of the plant, carefully washed and cleaned under a stereoscopic microscope (Olympus SZ 30) so as to remove all root pieces belonging to other plants. Samples were fixed in 70% formalin-acetic acid-alcohol (FAA).

Analyses of the samples.—All samples were stained using a modified Phillips and Hayman (1970) method (see Fernández *et al.*, 2008). For each individual three slides were made, each of them with ten stained root pieces of approximately 1 cm length mounted in glycerol. Root pieces were examined with a light microscope (Olympus BX40) at 20× magnification. The criterion used in this study for the determination of AM was the presence of arbuscules in at least one individual of each species. Structures corresponding to AM or other root fungi were documented as brightfield images, which were captured with a digital camera (Sony ExwaveHAD) and Image-Pro Plus 4.1.0.0. analysis software for Windows.

Results

A total of 66 sporophytes belonging to ten species in three families were sampled. Ten specimens (15%) corresponded to *Asplenium dareoides*, eight (12%) to *Grammitis magellanica* and 48 (73%) to various species of the family Hymenophyllaceae. In Puerto Blest, *A. dareoides* and *G. magellanica* are the only representatives of the Aspleniaceae and Polypodiaceae families respectively (Brion *et al.*, 1988; de la Sota *et al.*, 1998). At least five sporophytes were found for most of the species, except for *H. cuneatum*, *H. falklandicum* and *H. ferrugineum*, which were represented by only one or two specimens (Table 1).

Most of the samples (73%) were collected from epiphytic habitats and we observed that they frequently had soil particles attached to the outside of the roots, probably as a consequence of their proximity to the ground. Terrestrial sporophytes (27%) were collected from loose organic soils with abundant leaf and wood litter, 2 (11%) of them belonged to *A. dareoides* and 16 (89%) to five species of *Hymenophyllum* (Table 1). All the sporophytes examined presented extensive, thin (less than 2 mm wide) and fibrous root systems with abundant root hairs. Baylis (1975) and St. John (1980) described this root system morphology as "graminoid".

Most of the sporophytes lacked AM structures, but in seven terrestrial samples (11% of the total specimens analyzed), corresponding to *H. pectinatum* and *H. seselifolium* (Table 1), scarce knobby hyphae and structures similar to coils and vesicles were observed (less than 5% of the root length examined) (Fig. 2). However, arbuscules were not detected in any of these specimens, so it is questionable if these plants can be designated as mycorrhizal.

TABLE 1. Occurrence of arbuscular mycorrhizae in epiphytic and conspecific terrestrial sporophytes of ferns of Puerto Blest. **AM/Total:** number of individuals in which we observed AM / total of individuals examined, **(n°):** number of samples in which we observed structures similar to hyphal coils and vesicles, so it is doubtful whether these plants can be designated as mycorrhizal.

TAXON	LIFE FORM		AM/Total
	Epiphytic	Terrestrial	
Aspleniaceae			
Asplenium dareoides Desv.	8	2	0 / 10
Polypodiaceae			
Grammitis magellanica Desv.	8	0	0 / 8
Hymenophyllaceae			
Hymenophyllum cuneatum Kunze	0	1	0 / 1
Hymenophyllum falklandicum Baker	1	0	0 / 1
Hymenophyllum ferrugineum Colla	2	0	0 / 2
Hymenophyllum pectinatum Cav.	10	5	(4) / 15
Hymenophyllum seselifolium C Presl	4	7	(3) / 11
Hymenophyllum tortuosum Hook. & Grev.	5	2	0 / 7
Hymenophyllum umbratile Diem & JS. Licht.	4	1	0 / 5
Serpyllopsis caespitosa (Gaudich.) C Chr.	6	0	0 / 6

Abundant extra- and intracellular hyphae of regularly septate and generally melanized fungi frequently associated with microsclerotia and "cerebriform structures" were present in all root systems (Fig. 3). They corresponded to dematiaceous root endophytes usually named dark septate fungi (DSF).

DISCUSSION

Of the ten species examined in the present study, *G. magellanica* and *S. caespitosa* were found to be obligate epiphytes (only growing as epiphytes), while *A. dareoides* and five species of *Hymenophyllum* (*H. cuneatum*, *H. pectinatum*, *H. seselifolium*, *H. tortuosum* and *H. umbratile*) were facultative epiphytes (growing either epiphytically or terrestrially). These observations agree with de la Sota *et al.* (1998), who described the same habits for these species. We considered *H. cuneatum* as facultative epiphytic even though only one terrestrial sporophyte was collected because it is described as epiphytic in the literature (Rodríguez, 1995). *Hymenophyllum falklandicum* and *H. ferrugineum* could not be classified as obligate or facultative epiphytes because only one or two individuals per species were found (Table 1).

Representatives of the Aspleniaceae, Polypodiaceae and most of the Hymenophyllaceae families in Puerto Blest clearly lack AM colonization. Chaia *et al.* (2006) and Fernández *et al.* (2008) observed that different plant species growing in areas adjacent to our study site had AM. Furthermore, Chaia *et al.* (2006) found out that in Puerto Blest the AM soil infective capacity ranges from 0.6 to 1.7 IU g/soil. These findings indirectly demonstrate that

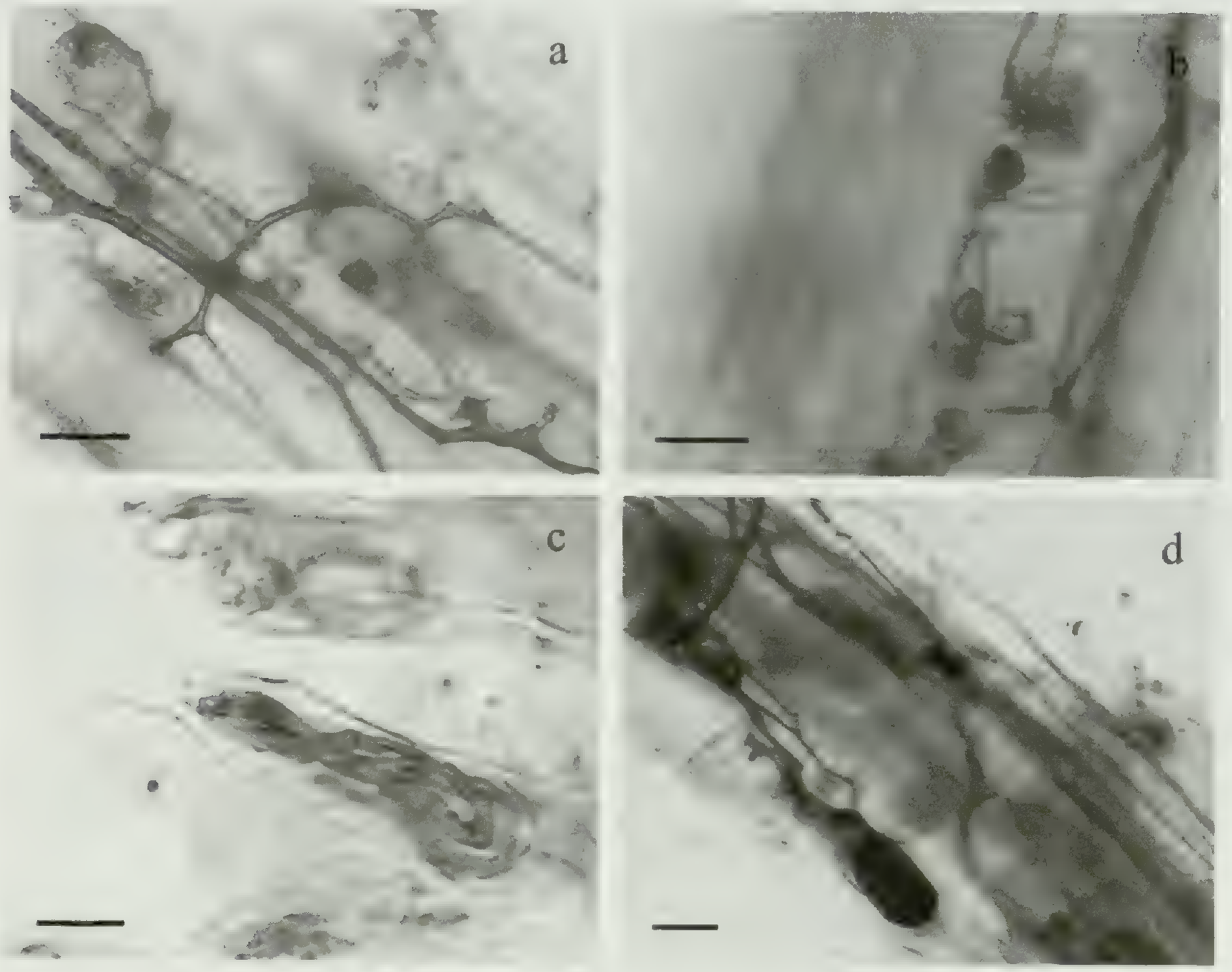

FIG. 2. Fungal colonization similar to arbuscular mycorrhizas in *H. pectinatum* (a,b) and *H. seselifolium* (c,d). **a:** Extra and intracellular mycelia where some septa can be observed, **b,c:** Structures resembling intracellular coils, **d:** Intracellular hyphae and a vesicle-like structure. *Scale bars*: **a,b**=20 μm; **c,d** = 50 μm.

there is AM inoculum in the soil where the samples were collected. However, terrestrial specimens of *A. dareoides*, *H. cuneatum*, *H. tortuosum* and *H. umbratile* did not form AM despite the presence of inoculum in the soil, which supports the non-mycorrhizal behavior of these species. Even though we found structures similar to vesicles and coils in roots of four out of five terrestrial sporophytes of *H. pectinatum* and in three out of seven terrestrial sporophytes of *H. seselifolium* (Fig. 2), it was not possible to record them as mycorrhizal because arbuscules (the diagnostic feature of the symbiosis) were absent and the colonization percentage was very low (less than 5%). Additionally, it is known that some other fungi are capable of forming structures similar to those of AM within roots of some epiphytic plants (Brundrett, 2002; Rowe and Pringle, 2005). These structures can also stain dark blue (Fig. 2c,d) and in consequence they may be mistaken for AM (Janos, 1993). The presence of such fungi could explain the presence of this type of structures in roots of *H. pectinatum* and *H. seselifolium*.

The absence of mycorrhizae in the epiphytic fern species examined in this work is concordant with previous studies that have analyzed this symbiosis in other species belonging to the same families (Cooper, 1977; Gemma *et al.*, 1992; Zhao, 2000; Wang and Qiu, 2006), but differs from some others that have

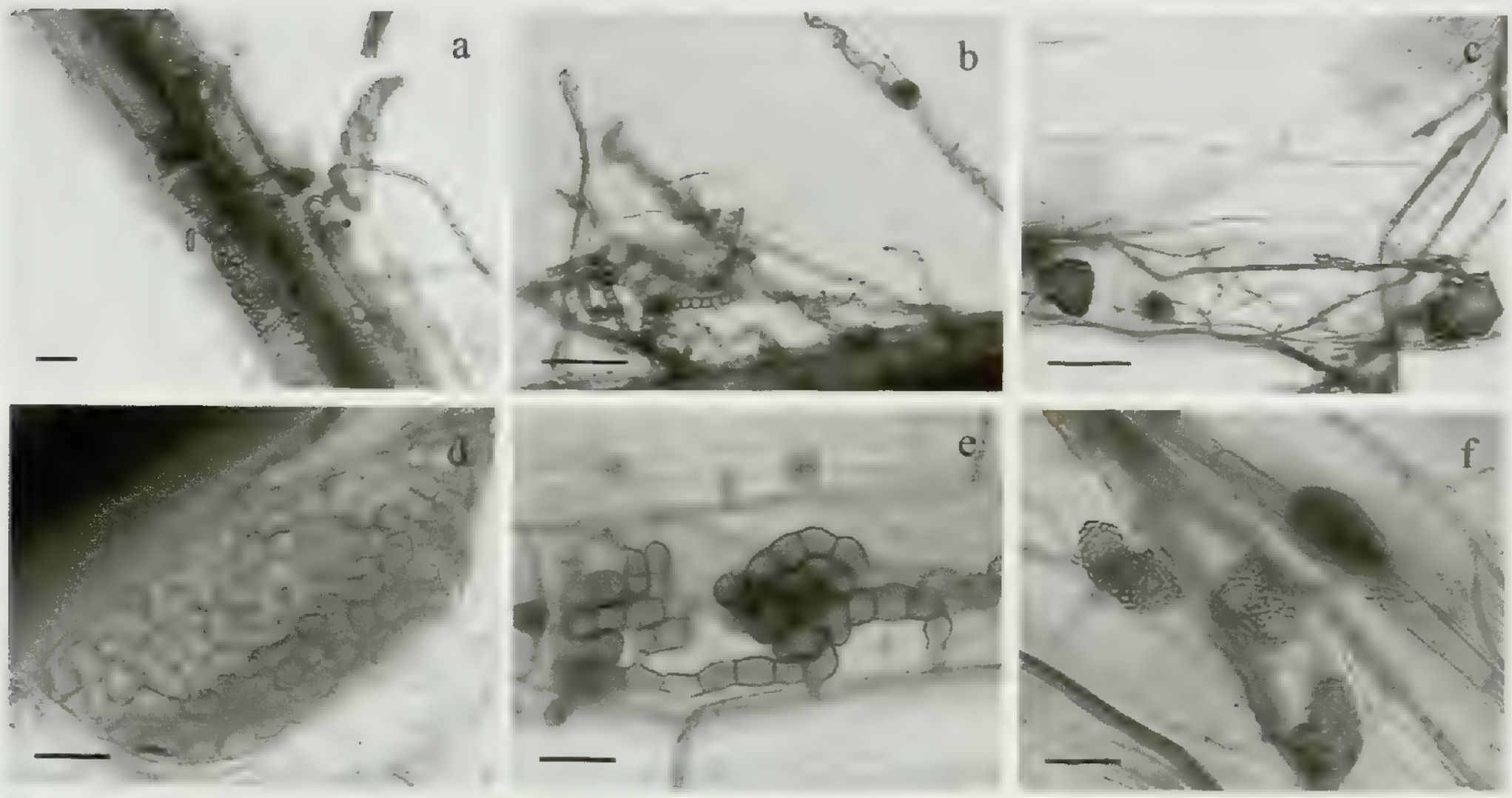

FIG. 3. Dark septate fungi in roots of epiphytic ferns of Puerto Blest. **a:** Extraradical hypha and intracellular microsclerotia in *S. caespitosa*, **b:** General view of extraradical DSF colonization in *G. magellanica*, **c:** Intracellular septate hyphae and associated vesicle-like structures in *H. ferrugineum* (structures stained blue; color not shown) **d:** Moniliform hyphae (structures stained blue; color not shown) within cortical cells of *A. dareoides*, **e:** Highly melanized moniliform hyphae in roots of *H. tortuosum*, **f:** Characteristic cerebriform microsclerotia in *H. seselifolium*. *Scale bars*: **a**=50 μm; **b,c**=100 μm; **d,e,f**=20 μm.

reported the occurrence of AM in the same group of plants (Cooper, 1976; Iqbal *et al.*, 1981; Gemma *et al.*, 1992; Muthukumar and Udaiyan, 2000; Zhao, 2000; Wang and Qiu, 2006). Our results are also consistent with Lesica and Antibus (1990) and Michelsen (1993), who examined 13 and eight species of epiphytic ferns in Costa Rica and Ethiopia respectively (including five species of *Asplenium* and one of *Hymenophyllum*), and described them as non-mycorrhizal. Nadarajah and Nawawi (1993) studied the mycorrhizal status of 19 fern species in Malaysia. They observed that all these species were non-mycorrhizal in epiphytic habitats and that eight of them were able to form AM vesicles and/or coils when they were rooted in soil. Nevertheless, as it occurred in this work, these authors did not detect arbuscules in any of the samples.

Although it has been suggested by several authors that AM are generally absent (Allen *et al.*, 1993) or rare (Bermudes and Benzing, 1989; Lesica and Antibus, 1990; Maffia *et al.*, 1993, 2000; Michelsen, 1993; Nadarajah and Nawawi, 1993) in epiphytes, Rains *et al.* (2003) found abundant mycorrhizal epiphytes in a Costa Rican cloud forest. This apparent discrepancy suggests that the occurrence, and possibly the role, of mycorrhizae in epiphytic habitats is variable and may be related to taxonomic affinities and diverse ecological factors (Tester *et al.*, 1987; Lesica and Antibus, 1990; Michelsen, 1993).

Different explanations have been suggested for the paucity of AM in epiphytic habitats. For example, it might be that these environments are too dry and exposed to support the fungus or that the rate of photosynthesis

(which is relatively low in epiphytes, see Benzing, 1986) may be insufficient to maintain the symbiosis (Lesica and Antibus, 1990). Nutritional insufficiency, and consequently the inability to sustain the energy cost of mycorrhizae, has also been suggested as a major factor determining mycorrhizal success in epiphytes (Benzing, 1990; Janos, 1993). Some substrate analyses have demonstrated that trunks and canopy mats might be nutrient rich (Bermudes and Benzing, 1989; Lesica and Antibus, 1990), and it is known that high fertility suppresses AM of facultatively mycotrophic species (Janos, 2007). Another widespread explanation is that epiphytic habitats are deficient in AM inocula, so epiphytes usually do not have the opportunity of being colonized by mycorrhizal fungi (Lesica and Antibus, 1990; Janos, 1993; Michelsen, 1993). This suggestion contrasts with several authors (McIlveen and Cole, 1976; Rabatin *et al.*, 1993; Janos *et al.*, 1995; Mangan and Adler, 2000) who have reported that AM spores and mycelia may be dispersed to epiphytic environments by several vectors (e.g., mainly birds, ants and small mammals). Moreover, other studies have established that many epiphytes lack AM despite the presence of spores and external mycelia in the substrate (Lesica and Antibus, 1990; Maffia *et al.*, 1993; Rabatin *et al.*, 1993).

In this study it was possible to compare the mycorrhizal status of six facultative epiphytic fern species. In the case of *A. dareoides*, *H. cuneatum*, *H. tortuosum* and *H. umbratile* all specimens lacked AM, and in most of the terrestrial sporophytes of *H. pectinatum* (73%) and *H. seselifolium* (73%) fungal structures similar to those of AM were completely absent. Therefore, deficiencies in AM inocula or in nutrient acquisition (which are not limiting factors when plants are rooted in soil, as has been demonstrated in previous studies carried out in Puerto Blest) would not explain the non-mycorrhizal behavior observed in these plants. Thus, it is necessary to seek other explanations for this phenomenon. Another factor that might explain the absence of AM in epiphytic ferns of Puerto Blest is root system morphology. Plants with long root length (Koide and Li, 1991; Ryser and Lambers, 1995), small root diameter (Reinhardt and Miller, 1990; Manjunath and Habte, 1991; Hetrick *et al.*, 1992) and abundant long root hairs (Baylis, 1975; St. John, 1980; Schweiger *et al.*, 1995), like those observed in this study ("graminoid roots"), tend to be non-mycorrhizal (Baylis, 1975; St. John, 1980; Hetrick, 1991; Michelsen, 1993; Zangaro *et al.*, 2005).

Dark septate fungi (DSF) are conidial or sterile fungi that have been reported in several plant species from different habitats (Jumpponen and Trappe, 1998; Jumpponen, 2001). Characteristic inter- and intracellular hyphae and microsclerotia corresponding to DSF were observed in every specimen analyzed in this work (Fig. 3). This is in accordance with previous studies that have reported the existence of this type of fungi within the roots of angiosperm epiphytes (Bermudes and Benzing, 1989; Allen *et al.*, 1993; Janos, 1993; Rains *et al.*, 1993) and several fern species (Cooper, 1976; Dhillion, 1993; Fernández *et al.*, 2008). The presence of "cerebriform microsclerotia" (Fig. 2f) has not been described in the literature before, except in *E. bogotense* and *L. paniculatum*, two facultative mycorrhizal species also studied in Puerto Blest

(Fernández *et al.*, 2008). The presence of DSF within the roots of different fern species demonstrates that these plants are capable of forming plant-fungal associations with a diversity of fungal lineages (Winther and Friedman, 2007). According to Allen *et al.* (1993) and Rains *et al.* (2003), the lack of AM in most of the epiphytes in addition to the occurrence of DSF in many of them is notable and may suggest that DSF may function as "mycorrhizal fungi", but further study is needed to test this hypothesis.

This study provides novel information about the occurrence of mycorrhizae in epiphytic ferns and it the first work in which the mycorrhizal status of epiphytic species of Patagonia, Argentina is recorded. More work on a larger scale is needed not only for understanding the behavior of epiphytic species of Puerto Blest but also for developing patterns of mycorrhizal activity in epiphytic habitats and for determining ecological explanations for the paucity of AM in these plants (Lesica and Antibus, 1990; Allen *et al.*, 1993; Nadarajah and Nawawi, 1993).

Acknowledgments

We are grateful to Parques Nacionales for giving us the opportunity to work in a protected area and we thank Dr. Davis Janos for critically reviewing our manuscript and for his valuable comments on this work. Funds for this research were provided by grant PICT 22200 (FONCyT).

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American Fern Journal 100(1):27–31 (2010)

The Cuticle on Gametophytes of *Tmesipteris sigmatifolia*

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ABSTRACT.—The surface of gametophytes of *Tmesipteris sigmatifolia* from axenic culture were examined with electron microscopy and histochemistry. A lipid layer, which gave a positive reaction with lipid stains, covers the surface of these gametophytes. In apical regions of the gametophyte the lipid coating, the cuticle, is less than half the thickness of the wall it covers. The wall was not stained with lipid stains, but did stain for cellulose and polyphenolic materials. A cuticle on the surface of *Tmesipteris* gametophytes indicates that a cuticle is characteristic of gametophytes in the Psilotaceae because gametophytes of both *Psilotum* and *Tmesipteris* are now known to have them.

KEY WORDS.—*Tmesipteris*, gametophyte, cuticle

The gametophytes of the Psilotaceae are different from the typical photosynthetic fern gametophytes because they are subterranean, mycorrhizal, and long-lived. They are cylindrical gametophytes that often branch (Holloway, 1917; Lawson, 1917b). Their surface is of interest because these gametophytes grow in soil or humus for long periods of time.

Early studies reported the cuticularization of the surface of *Psilotum* gametophytes (Darnell-Smith, 1917; Holloway, 1939) and Bierhorst (1971) demonstrated lipid on their surface using Sudan IV. The structure of the surface wall of these gametophytes, including the lipid materials, was examined with histochemical techniques and electron microscopy by Whittier and Peterson (1995). It was concluded that the surface lipid layer is best considered a cuticle.

Less is known about the surface of *Tmesipteris* gametophytes. Holloway (1917) illustrated the surface wall as being thicker than the inner walls of these gametophytes and Lawson (1917a) commented on and illustrated this thicker wall. Neither study reported the cuticularization of the gametophyte surface. Because a lipid layer was not demonstrated in these studies, the present study was initiated to determine if lipids are associated with the surface of *Tmesipteris* gametophytes.

MATERIALS AND METHODS

Gametophytes of *Tmesipteris sigmatifolia* Chinnock were grown on 25 ml of nutrient medium in 25 × 150 mm culture tubes with screw caps, which were tightened to reduce moisture loss. The nutrient medium contained 100 mg NH_4Cl, 100 mg $MgSO_4 \cdot 7H_2O$, 40 mg $CaCl_2$, and 100 mg K_2HPO_4 per liter. In addition, a liter of medium contained 4 ml of a FeEDTA solution (Sheat *et al.*, 1959), 0.5 ml of a minor element solution (Whittier and Steeves, 1960), and 5 g of glucose. The medium was solidified with 1.1% agar and was at pH 6.0 before autoclaving. The gametophytes were cultured in the dark at 23 ± 1°C until used.

For transmission electron microscopy (TEM) the gametophyte tissue was fixed overnight on ice in a 1:1 solution of 4% glutaraldehyde and 10% acrolein in 0.07M phosphate buffer (pH 6.8). The tissue was postfixed in 1% osmium tetroxide in 0.07M phosphate buffer (pH 6.8) on ice for 2 hr, followed by dehydration in a graded acetone series and embedded in Spurr's resin (Spurr, 1969). Thin sections were picked up on stainless steel grids and stained with a saturated solution of uranyl acetate in methanol followed by lead citrate (Venable and Coggeshill, 1965).

A variety of methods were used to prepare the gametophyte material for light microscopy. Hand sections or pieces of living gametophytes were employed for some histochemical tests. Thick sections (0.5 μm) of gametophytes in Spurr's resin, as prepared for TEM, were used for other tests.

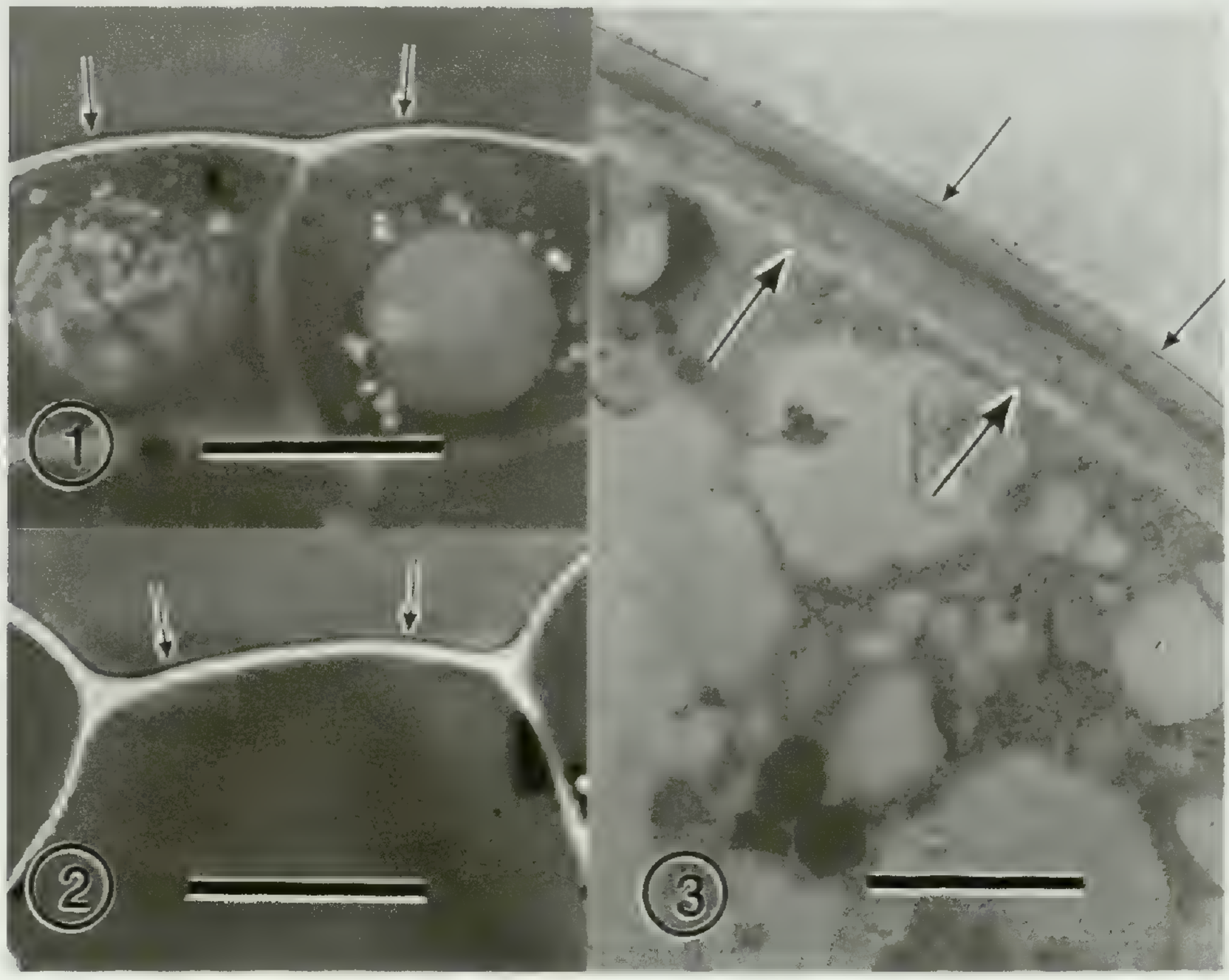
To determine the presence of lipids, hand sections were stained with Sudan IV (Jensen, 1962). Thick sections of gametophytes in Spurr's resin were stained for lipids with Sudan black B (Bronner, 1975). Lipids were extracted from the Spurr's material with the technique of Eurenius and Jarskar (1974) and from hand sections with a warm methanol-chloroform (1:1) mixture (Pearse, 1968). The IKI-sulfuric acid test (Jensen, 1962) was used to demonstrate cellulose and the ferric-ferricyanide test (Wilson and Peterson, 1983) was employed to show polyphenolic materials.

Resistance of a cell wall to concentrated sulfuric acid digestion is characteristic of walls containing cutin, suberin, or lignin (Wilson and Peterson, 1983). Gametophytes were digested with sulfuric acid for 18 hr. After this treatment, they were stained with Sudan IV for evidence of lipid. The phloroglucinal-HCl test (Jensen, 1962) was employed in an attempt to demonstrate lignin in these walls.

Results

The surface of *Tmesipteris* gametophytes has two layers (Figs. 1 and 2) and the outer layer is lipid material. This layer is observed in material embedded in Spurr's resin that has been sectioned and stained with Sudan black B (Figs. 1, 2). Hand sections of living gametophytes show the lipid layer stained with Sudan IV. In the preparations using the Sudan stains, the inner layer did not stain, although lipid droplets in the cytoplasm did (Fig. 1 and 2). The inner layer of the surface stains for cellulose and polyphenolics. Crushing the wall increased the cellulose staining with IKI-sulfuric acid. The outer lipid layer did not stain with the stains for nonlipid materials.

Distinct layers to the gametophyte surface can be seen with transmission electron microscopy (Fig. 3). Young gametophyte cells have two thick layers making up the surface (Fig. 3) and the outer lipid layer averaged about 30% of the surface thickness. The lipid layer is electron lucent and primarily amorphous and it abuts directly on the inner polysaccharide layer. There is a thin line of darker material at the outer surface of the polysaccharide layer that is not evident with light microscopy.



FIGS. 1–3. Light and electron microscopy of the surface of *Tmesipteris* gametophytes. **1.** Sudan Black B staining of the gametophyte surface (arrows) and small lipid droplets in the cells. The polysaccharide portion of the surface wall and starch grains in the cells are unstained and white. **2.** Sudan black B staining of the gametophyte surface (arrows) and the unstained portion of the surface wall. **3.** The two-layered gametophyte surface, as demonstrated with electron microscopy, having an outer electron-lucent layer (small arrows) and an inner polysaccharide layer (large arrows). Scale bars in Figs. 1–2 = 30 μm and Fig. 3 = 2 μm.

Efforts were made to dissolve the outer lipid layer on the gametophyte surface. The technique of Eurenius and Jarskar (1970) removed lipid droplets from the cells embedded in Spurr's resin, but the outer lipid layer remained and stained with Sudan black B. Warm methanol-chloroform removed lipid from the cells in hand sections, but not the lipid staining material from the gametophyte surface. Treatment of gametophytes with sulfuric acid overnight did not digest the outer wall of the gametophytes. They remained intact, although the cell contents were destroyed. The lipid layer could still be stained with Sudan IV. The failure of the surface walls to be digested is an indicator of cutin, suberin, or lignin. A negative phloroglucinol-HCl test demonstrated the absence of lignin.

DISCUSSION

The earlier studies of Holloway (1917) and Lawson (1917a) did not report lipid on the surface of *Tmesipteris* gametophytes. However, the techniques (Whittier and Peterson 1995), which confirmed the presence of a lipid coating on *Psilotum* gametophytes, proved useful in studying the surface of *Tmesipteris* gametophytes. Positive staining of the gametophyte surface with

Sudan black B and Sudan IV and observations with electron microscopy demonstrated a lipid coating on *Tmesipteris* gametophytes. This layer appears to be the same as the lipid layer of *Psilotum* gametophytes.

Resistance of the gametophyte surface to sulfuric acid (Wilson and Peterson, 1983) and failure of organic solvents to extract the fatty material (Holloway, 1982b; Kolattukudy *et al.*, 1981) is an indication that cutin or suberin is present. Whether the coating is composed of cutin or suberin is unknown at this time. Simple staining procedures cannot distinguish one from the other (Wilson and Peterson 1983). The elaborate chemical analyses necessary to identify them (Kolattukudy, 1984) are beyond the scope of this study.

The surface wall of *Tmesipteris* gametophytes does not have the appearance of a suberized wall. The lipid layer is on the outer surface and not in the wall next to the plasma membrane, as would be expected for a suberized wall. Also, if the lipid material is suberin, it should have stained with stains for phenolic materials (Scott and Peterson, 1979), which it did not.

The lipid layer of these gametophytes is more similar to a cuticle than to a suberized wall. Its position as a surface layer on the external wall is typical of a cuticle. The amorphous structure of this layer is similar to that identified as a type 6 cuticle by Holloway (1982a). The lipid layer on *Tmesipteris* gametophytes, although possibly thinner, is basically the same as that on *Psilotum* gametophytes. It should be considered a cuticle until proven otherwise by critical chemical analyses.

Finding a cuticle on the surface of *Tmesipteris* gametophytes indicates that a gametophytic cuticle is characteristic of the Psilotaceae because gametophytes of both genera are now known to have them. It is not surprising that *Tmesipteris* gametophytes have a cuticle because they grow under the same or similar conditions as the rhizome of *Tmesipteris* and the gametophytes of *Psilotum*, both with cuticles. Both the gametophytes and rhizomes of *Tmesipteris* grow slowly in soil or humus and are long-lived. A cuticle would help to prevent the gametophytes from drying out, if there was drying of the soil or humus. Besides resistance to drying, the cuticle could help to protect the gametophytes from various biotic factors in their habitat. The occurrence of a cuticle on these gametophytes under the relatively moist conditions of axenic culture indicates that this is a permanent feature of these gametophytes and not just a response to drier conditions.

Acknowledgments

I thank Lewis Melville from the laboratory of R. L. Peterson at the University of Guelph for technical assistance with the electron microscopy.

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American Fern Journal 100(1):32–38 (2010)

Gametophyte of the Andean Fern *Cheilanthes pilosa* Goldm. (Pteridaceae)

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Abstract.—The gametophytic generation of *Cheilanthes pilosa* (Pteridaceae), including spore germination, morphological development of the gametophytes, major vegetative features and sexual expression, was studied. In *C. pilosa* spore germination was of the *Vittaria* model and the developmental pattern was intermediate between *Adiantum* and *Ceratopteris* models. Adult gametophytes were cordate and hairy, with unicellular hairs located in the margins and both the ventral and dorsal surfaces of the prothalli. Gametangia were of the normal type described for leptosporangiate ferns. In *C. pilosa* populations the gametophytes produced at first instance a high proportion of female gametophytes, few male gametophytes developed and most of the female gametophytes became bisexual with time. Thus, although outbreeding is possible, this species seemed to be promoting intragametophytic selfing as the major reproductive strategy.

Key Words.—gametophyte, development, reproduction, Andes, Peru, *Cheilanthes pilosa*

Cheilanthes Sw. (Pteridaceae) is a large genus that comprises around 150–200 species. It is a cosmopolitan genus, but most species inhabit semi-arid locations of tropical and subtropical areas (Tryon, 1990). The genus presents three major biodiversity centers (Tryon and Tryon, 1973): one in America (from the USA-Mexico zone, where it is very abundant, until the Andes), another in Australia and the third in the Mediterranean basin, especially Greece and the Iberian Peninsula. It also appears in SE Africa. *Cheilanthes pilosa* Goldm. is a saxicolous species that occurs in the Andean areas of Peru and Bolivia.

The gametophytic phase of *Cheilanthes* has been subject of abundant literature. Among others, apogamy (Whittier, 1965; 1970), physiology and ecophysiology (Quirk and Chambers, 1981; Nondorf *et al.*, 2003; Palmieri and Swatzell, 2004) and morphology and development (Pray, 1961; Nayar, 1963; Pangua and Vega, 1996; Gabriel y Galán and Prada, 2009) have been studied in the genus. The aim of this work was to study spore germination, the prothallial development, the morphology of the adult gametophyte and the sexual behavior of *C. pilosa*.

Material and Methods

Spores for cultures were taken from sporophytes collected in Peru, Cuzco Department, Urubamba Province. The following is the location of the

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collection: Ollantaytambo, pasado Huilloq, 13°14′50.5″S 72°15′28.8″W, 2.920 m, roquedo, Gabriel y Galán s/n, 28/04/2008. The material was identified following Tryon and Stolze (1989). Vouchers are deposited in MA.

Each spore sample for cultures were taken from separate sporophytes kept dry at room temperature for around 8 months. Gametophytes were grown under fluorescent light on a 12-h light, 12-h dark cycle at 20 ± 2°C, in 6 cm plastic Petri dishes. Multispore cultures on mineral agar medium (Dyer, 1979) were established by sacking fertile pinnae on a weigh paper, and placing the obtained spores in the Petri dishes. The sowing was replicated twice for each sample. Percentage of germination was recorded on every third day for a random sample of 50 spores from each of the two plates, until the maximum germination was reached. Spore sizes given are the mean of 30 measurements. To study the stages of gametophyte development, random samples were taken weekly, from the beginning of spore germination until sexual maturity. Gametophytes were stained with chloral hydrate acetocarmine (Edwards and Miller, 1972), mounted in water and observed under a light microscope. Some in vivo observations were also made.

Results

The spores of *C. pilosa* were trilete, spherical, 39.2 µm in polar view, and presented the perispore with prominent cristae that tended to form a net of irregular areolae. Germination started about 5–7 days after sowing, when few spores (ca. 2%) showed a first rhizoid. Later, the first prothallial cell emerged, perpendicularly to that of rhizoid. After the first 7 days in culture, germination increased rapidly, reaching ca. 60% in the following 7 days and a maximum of 83% during the next week. Figure 1 shows the progress of the germination percentages over the days considered.

The development of the gametophyte of *C. pilosa* was very quick. Three to four days after germination, short filaments of 3–4 cells (ca. 150 µm in length) were formed (Fig. 2A). The apical cell underwent two oblique longitudinal divisions that produced 3 daughter apical cells. In one of them, a new division led to the formation of a first hair (Fig. 2B). Following transversal and longitudinal divisions in the apical and in the 2–3 subapical cells, formed a spatulate gametophyte of about 4–5 cells broad. The first 2–4 hairs developed in an alternate way in both the margins (Fig. 2C). Around 15 days after germination, these spatulate gametophytes organized an apical meristem by longitudinal divisions in the apex (Fig. 2D), which led to the development of a pre-cordate prothallus (Fig. 2E). At this stage, ca. 25–30 days after germination, the wings were beginning to develop, while the margins of the plate continued forming hairs (Fig. 2F), some of them as culmination of marginal projections. In ca. 40 days after germination, adult gametophytes were completely developed. These gametophytes were broad, of about 2 mm in width, hairy and symmetrically cordate (Fig. 2G).

The rhizoids of *C. pilosa* had the capacity of abruptly changing the direction of elongation, by causing the subapical region to grow in another direction

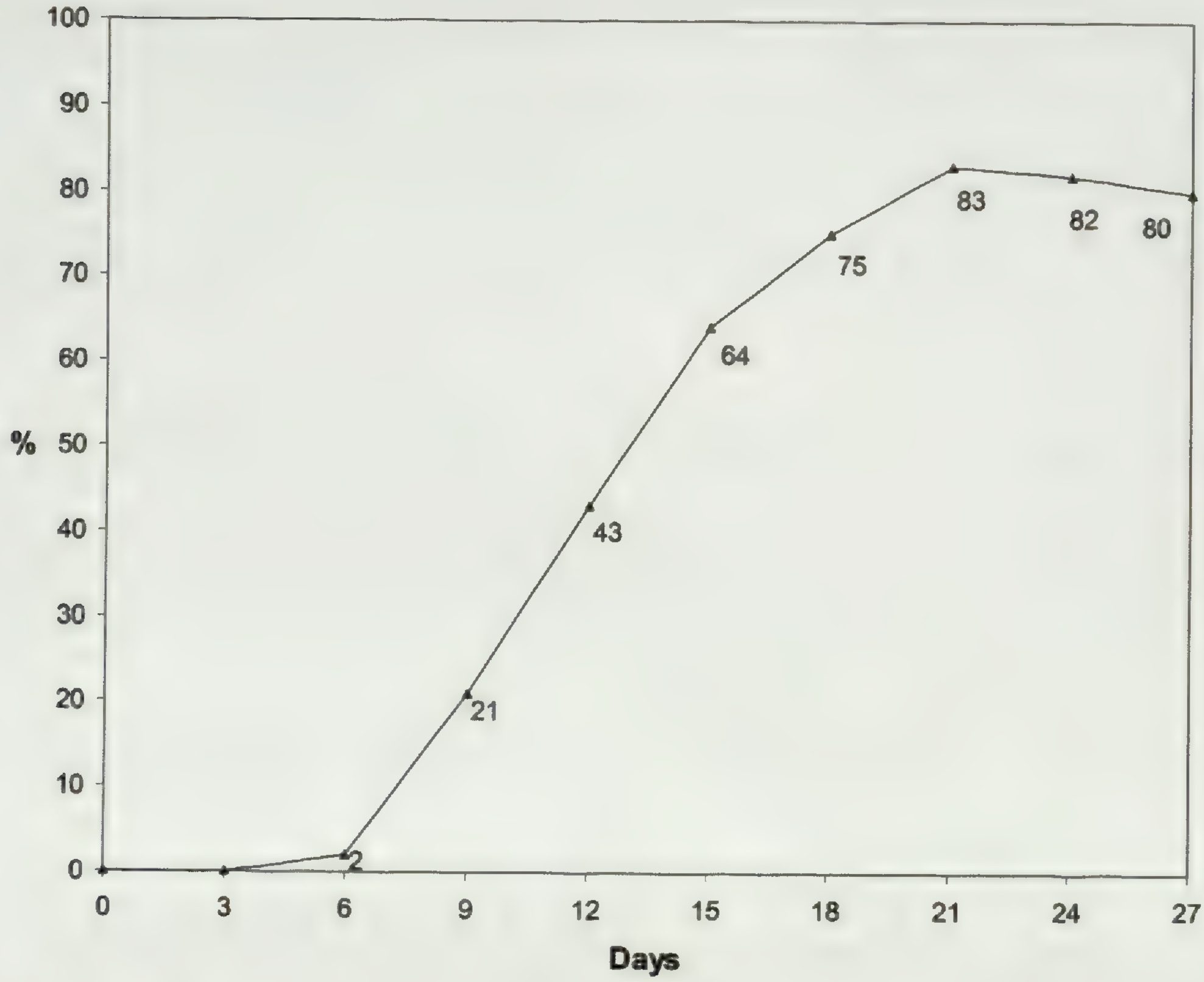

FIG. 1. Changes in the germination percentages of *Cheilanthes pilosa*, over 27 days.

from the apical region (Fig. 3A). In young and adult gametophytes of *C. pilosa*, numerous unicellular hairs could be seen (Figs. 2C–G), located in the margin and in both dorsal and ventral surfaces. The size of the hairs was ca. 50 μm. The marginal ones were subtended by a somewhat spindle-shape cell (Fig. 3B).

Cheilanthes pilosa developed gametangia 40 days after germination. Firstly, archegonia were relatively abundant, present immediately behind the apical notch. Female gametophytes were the only fertile ones in the cultures for a period of around 20 days, reaching ca. 60% of the prothalli; the rest were sterile. Later, some female gametophytes began to produce antheridia and became bisexual. Antheridia developed less frequently, and were located in the vicinity of the archegonia (Fig. 3C). For the rest of the observation period (ca. 250 days) bisexual prothalli became predominant in the cultures, reaching ca. 60%. Nevertheless, ca. 30% of the gametophytes were always female and ca. 10% were sterile. Strictly unisexual male gametophytes were observed occasionally, but unlike the bisexual ones, developed antheridia in great number (Fig. 3D).

DISCUSSION

The spores of *C. pilosa* show sizes and perispore ornamentations that fall in the normal ranges of variation and types previously reported for the genus

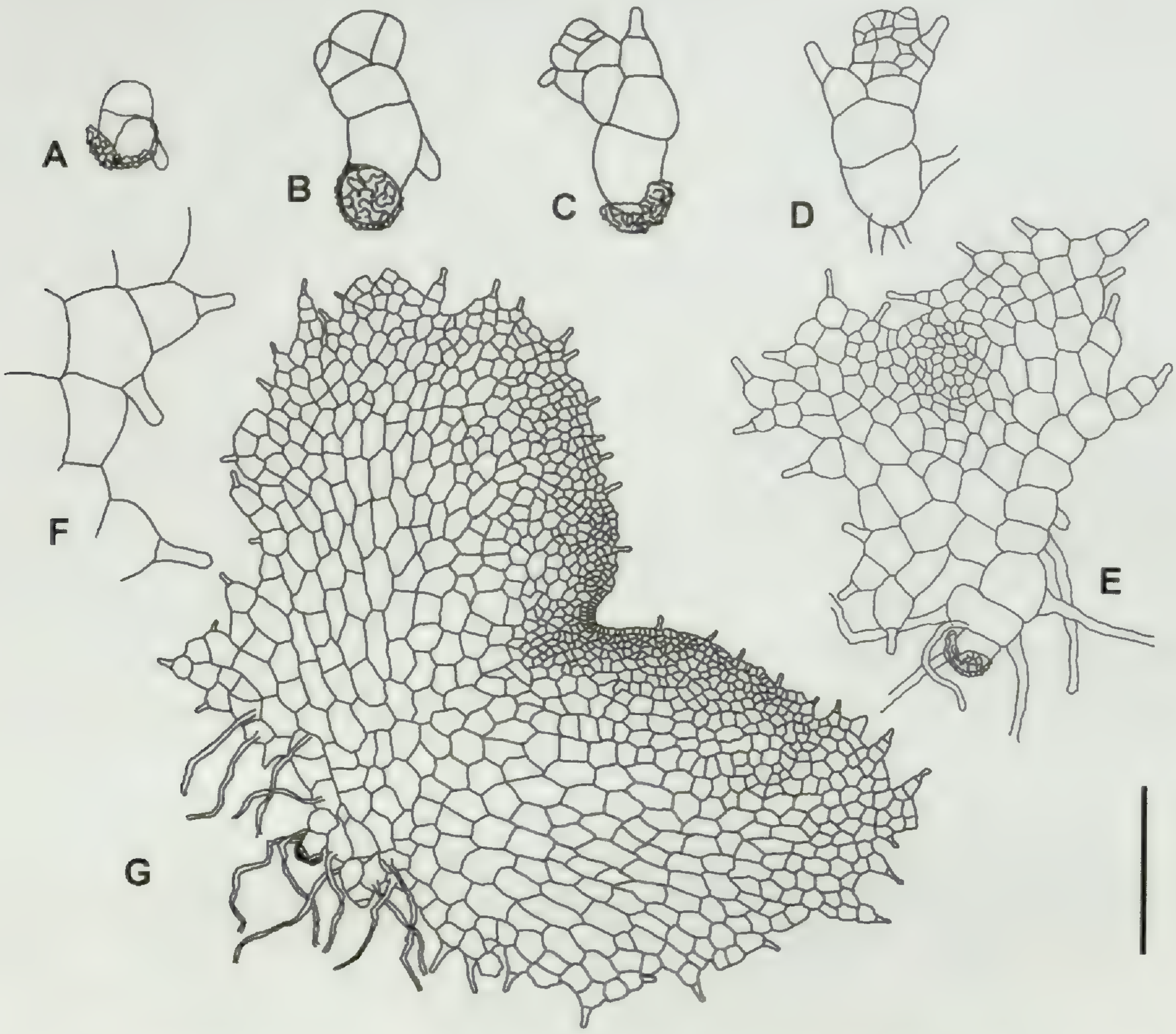

FIG. 2. Gametophyte development of *Cheilanthes pilosa*. A: Initial filament stage, 3 days. B: 3-celled filament with first longitudinal divisions in apical cell, 7 days. C: spatulate gametophyte with production of first hairs, 10 days. D: spatulate gametophyte with the beginning of the apical meristem, 15 days. E: initial cordate stage, 28 days. F: detail of marginal hairs, 28 days. G: adult prothallus, 40 days. Days are measured from the germination. Bar = 100 μm in F; 180 in A–D; 250 μm in E; 420 μm in G.

(Tryon and Lugardon, 1990). The spore germination is of the *Vittaria* model, in which the first rhizoid and the first prothallial cell emerged perpendicularly one to each other. This is the most common way of germination in the leptosporangiate ferns (Nayar and Kaur, 1968) and has been reported in other *Cheilanthes* species (Gabriel y Galán and Prada, 2009).

Gametophyte developmental process in *C. pilosa* is intermediate between the *Adiantum* model, in which an apical meristematic cell is formed in early stages, and the *Ceratopteris* model, in which the meristem is developed in a bidimensional plate from marginal cells (Nayar and Kaur, 1969). In *C. pilosa*, the meristem is formed in a spatulate bidimensional prothallus but from an apical cell. This kind of intermediate condition has been previously reported from other *Cheilanthes* species, as *C. aemula* Maxon, *C. leucopoda* Link and *C. meifolia* Eaton (Nayar and Kaur 1969), *C. tinaei* Tod. and *C. acrostica* (Balb.)

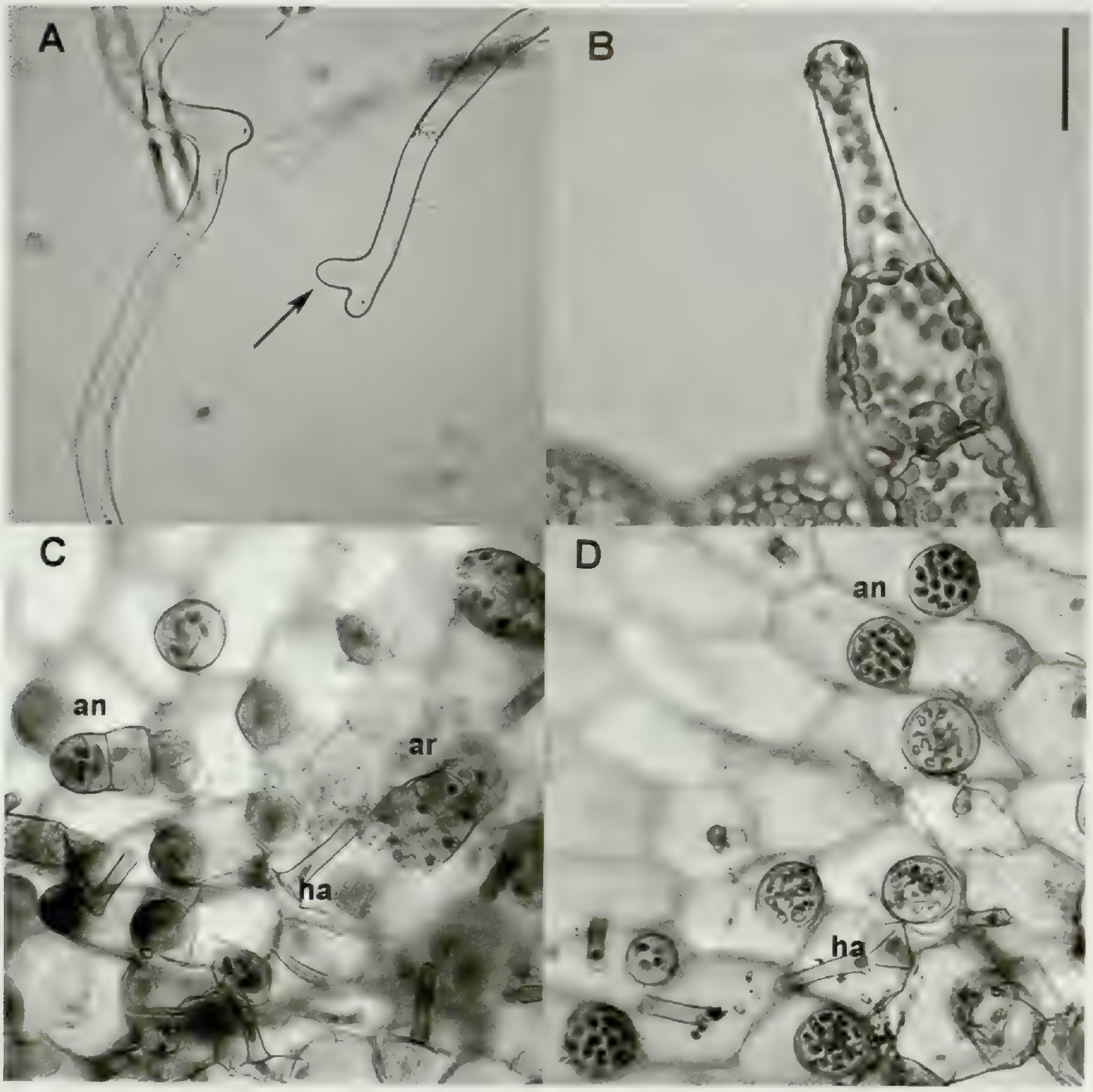

FIG. 3. Vegetative and reproductive features of *Cheilanthes pilosa*. A: rhizoids, showing subapical elongation (arrow), on a young cordate prothallus, 30 days. B: marginal hair, subtended by a spindle-shape cell, on an adult sexual prothallus, 50 days. C: gametangia on bisexual gametophyte, 80 days. D: antheridia on male gametophyte, 70 days. an=antheridium; ar=archegonium; ha=hair. Days are measured from the germination. Bar = 35 μm in A; 20 μm in B, C; 15 μm in D.

Tod. (Pangua and Vega 1996), and *C. glauca* (Cav.) Mett. (Gabriel y Galán and Prada, 2009). It seems to be the normal way of gametophyte development in the genus, while the typical *Adiantum* and *Ceratopteris* models are uncommon (Nayar & Kaur 1971). In addition, unlike most of the above mentioned species, *C. pilosa* formed marginal hairs from early stages of the development process.

In general, *Cheilanthes* is known for producing hairless gametophytes. Atkinson (1973) stated that, among the Pteridaceae, the majority of genera present hairless gametophytes, and those with hairy gametophyte are confined to the *Notholaena* group. Nayar and Kaur (1971) said that all genera in the Cheilanthaceae are naked, except for *Notholaena*, although some of the

examples they used are now considered under *Cheilanthes* (*N. galeottii* Fée, accepted name *Cheilanthes galeottii* (Fée) Mickel & Beitel). We also know of the naked gametophytes of *C. alabamensis* (Buckley) Kunze (Whittier, 1965), *C. tinaei* and *C. acrostica* (Pangua and Vega, 1996) and *C. glauca* (Gabriel y Galán and Prada, 2009). In addition, there are gametophytes of *Cheilanthes* (Giauque, 1949; Nayar and Kaur, 1971) and *Notholaena* (Tryon, 1947; Nayar and Kaur, 1971; Atkinson, 1973) that are known to be glandular, even producing wax-secretions. Currently, the presence of wax-farinose glands on gametophytes is considered to be a synapomorphy of *Notholaena* (Rothfels *et al.*, 2008). In conclusion, *Cheilanthes* presents naked gametophytes as the rule but also rarely hairy gametophytes, like *C. galeottii* (hairs 2-celled) and *C. pilosa* (hairs unicellular). A reexamination of the information on the gametophytes of the group is necessary to understand if gametophyte characters can be used to support the modern revisions of the family (Gastony and Rollo, 1995; Schuettpelz *et al.*, 2007).

The sex organs in *C. pilosa* were of the normal type defined for the leptosporangiate ferns (Nayar and Kaur, 1971). Although it has been said that the normal sequence of gametangia development in the lepotsporangiate ferns begins with the antheridia and continues with the archegonia (Atkinson and Stokey, 1964; Nayar and Kaur, 1971), there are many deviations reported. That is the case of *C. pilosa*. The great initial production of female gametophytes in *C. pilosa* coincides with other species, as *C. tinaei* (Pangua and Vega, 1996), but differs from others, as *C. acrosticha* (Pangua and Vega, 1996) and *C. glauca* (Gabriel y Galán and Prada, 2009), in which the sexual expression begins with a great proportion of male gametophytes. The scarce number of male gametophytes in the populations of *C. pilosa* is indicative of a low rate of intergametophytic unions, while the increasing development of bisexual prothalli from the females promotes the intragametophytic reproduction. The occurrence of this last strategy is also supported by the very close location of both types of gametangia.

Acknowledgments

The authors thank the financial support for the field trip to the Spanish Red Universitaria de Investigación sobre Cooperación para el Desarrollo (project G07/10).

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American Fern Journal 100(1):39–44 (2010)

Cyathea srilankensis Ranil (Cyatheaceae): A New Tree Fern Species From Sri Lanka

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Abstract.—*Cyathea srilankensis* Ranil, a new endemic Sri Lankan tree fern species, is described. It is characterized by large pinnate-pinnatisect leaves, sparse narrowly triangular petiole scales and caducous membranous indusia.

Key Words.—*Cyathea srilankensis*, endemic, tree ferns, Sri Lanka

The tree fern family Cyatheaceae consists of about 700 species worldwide with diversity centers in tropical wet mountain regions (Tryon and Gastony, 1975; Conant *et al.*, 1996). The family Cyatheaceae is one of the most interesting families among the ferns, due to its wide geographical distribution combined with pronounced local endemism (Tryon and Gastony, 1975). In Sri Lanka, the family is represented by a single genus, *Cyathea* Sm., which includes five species, one variety and one suspected hybrid (Holttum, 1965a; Sledge, 1982; Ranil *et al.*, 2004; and Philcox, 2006). During recent fieldwork in the Sinharaja forest reserve four mature individuals of *Cyathea srilankensis* Ranil were observed together with a small population of immature plants. All individuals were clustered in a single locality. Specimens collected from four mature and four juvenile plants were used to describe the species.

Cyathea srilankensis Ranil, ***sp. nov.*** TYPE.—Sri Lanka. Sinharaja forest: Prov. Sabaragamuwa, Ratnapura District, 06°41′664″N, 80° 42′380″E, 542 m elevation, 15 May 2007, *R.H.G. Ranil* 50R (holotype: PDA, 5 sheets). **Fig. 1 A–H** and **Fig. 2 A–E**.

Filix arborescens. Truncus usque ad 210 cm. altus, usque ad 5 cm. crassus, basibus petiolorum emortuorum vestitus. Petiolus 18–28 cm. longus, prope basim 0.8–1.0 cm crassus. Paleae petioli 0.8–1.2 cm. longae, 0.1–0.15 cm. latae, anguste triangulares, atrocastaneae, apice spina prominente sordide brunnea provisae. Lamina pinnato-pinnatisecta, 130–202 cm . longa, 40–65 cm. lata. Pinnae 15–30 cm. longae, 2–3 cm. latae, 44–57 iugatae. Pinnulae 1.5–2 × 0.5 cm.,

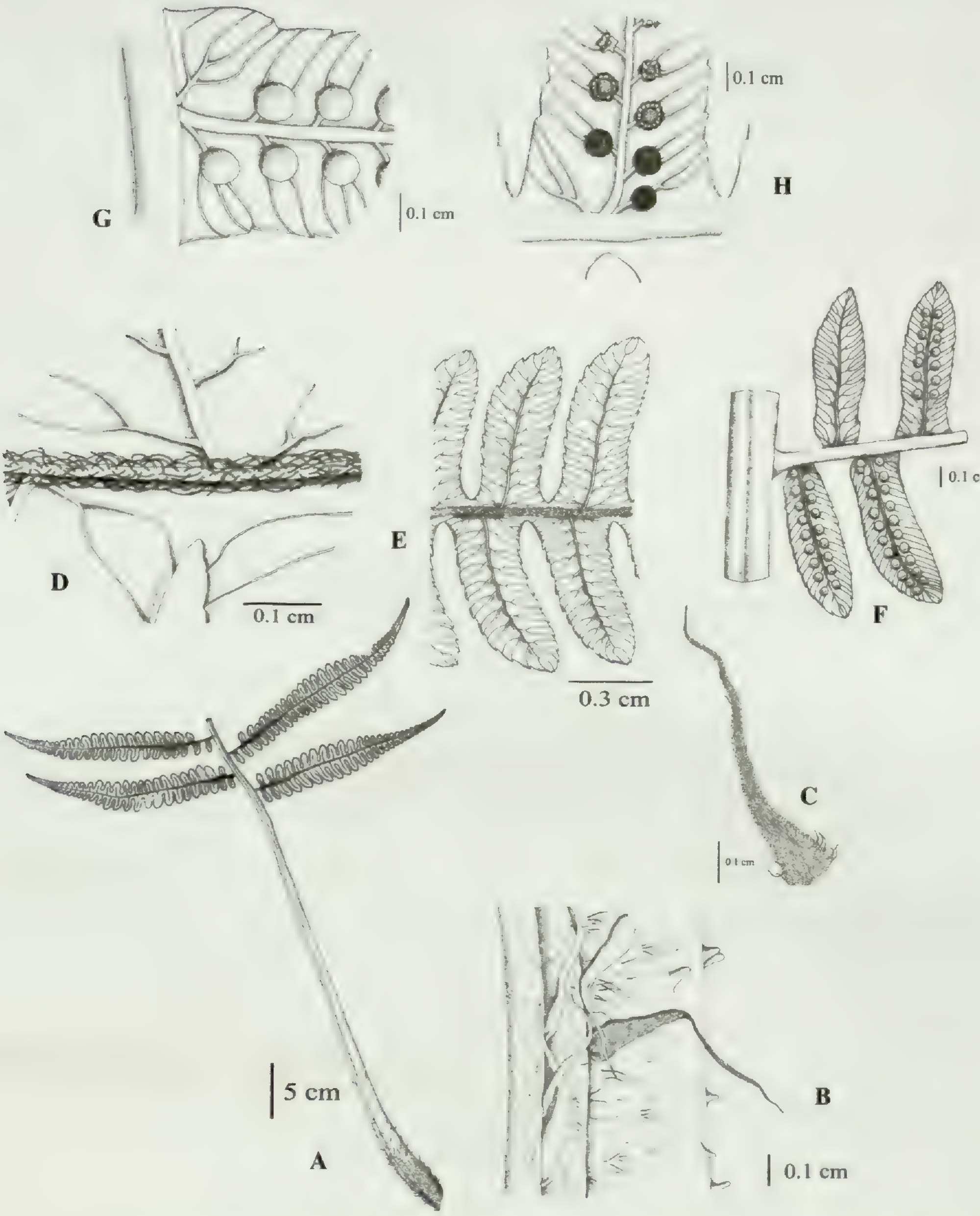

FIG. 1. *Cyathea srilankensis* Ranil. **A.** Petiole with scales and pinnae, **B.** A part of the petiole with scales and spines (adaxial face), **C.** Petiole scale, **D.** Adaxial face of the costa with dense hairs, **E.** Part of a pinna with broadly adnate pinnules (adaxial face), **F.** and **G.** Pinnules with immature sori, **H.** Pinnule with different maturity stages of sori.

ad costam adnatae (iuga infima sessilia) et pinnulae contiguae confluentes. Sori indusiis membranaceis, hyalinis, in statu juvenili globose, sed cito emarcidis, in soris maturis solum rudimenta exhibentibus (collum basi receptaculi).

Trunk erect, unbranched, up to 210 cm in height, 5 cm in diameter, entirely covered by persistent dead petiole bases, leaf scars not visible, usually with

FIG. 2. *Cyathea srilankensis* Ranil. **A.** Umbrella shaped crown; **B.** Lower surface of the leaf with sori; **C.** Trunk apex with petiole base; **D.** Trunk surface covered by persistent leaf bases; **E.** Trunk with adventitious shoots.

few adventitious shoots. Crown of 4–8 large leaves crowded at the apex of the stem. Petiole dark brown to dark chestnut coloured, shiny, 18–28 cm long, 0.8–1.0 cm in diameter at its base, with blunt, 0.1–0.2 cm long spines, dense at the petiole base. Petiole scales 0.8–1.2 cm long, 0.1–0.15 cm wide at their base, coriaceous, linear to narrowly triangular, with an apical spine, margin sometimes with several conspicuous dark setae, scales densely covering the younger leaves. Lamina large, 130–202 × 40–65 cm, pinnate-pinnatisect, elliptic to lanceolate, dark green adaxially, pale green abaxially, with a pinnatifid apex, subcoriaceous to coriaceous, rachis dark brown and shiny, glabrous adaxially, few lower pinnae at the base, abruptly narrowed and more sparsely placed. Pinnae 15–30 × 2–3 cm, 44–57 pairs, the lowest pinnae 15–20 cm long, the middle pinnae 15–30 cm long, sessile or shortly petiolulate (petiolule 0.2–0.3 cm long), alternate, linear, their apex pinnatifid, serrate, acuminate, middle pinnae 4–5 cm apart. Costa densely hairy adaxially, dark brown, glabrous. Pinnules 1.5–2 × 0.5 cm, 30–40 pairs, 0.2 cm apart, basal pinnule pair free, others broadly adnate to the costa and adjacent pinnules confluent from the lower quarter of the pinna, alternate, oblong and falcate, with an obtuse to acute apex, margin serrate. Veins once or twice forked, the proximal pair three or four times forked, 8–10 pairs in each pinnule, free, reaching the margin. Sori round, about 0.1 cm in diameter, 8–10 pairs in each

pinnule in two parallel rows on either side of the costula, inserted close to the costula at the fork of the veins, indusiate. Indusia globular, light brown, very thin, membranous, gradually degraded, at maturity only a reduced uneven disc around the base of receptacle persists. Spores trilete.

DISTRIBUTION.—Endemic to Sri Lanka and most likely limited to the Sinharaja forest reserve at Ratnapura district in Sabaragamuwa Province.

ECOLOGY.—All plants were recorded from roadside banks and open areas in Sinharaja forest associated with sun loving plants, such as *Clidemia hirta* (L.) D.Don, *Melastoma malabathricum* L., *Hedyotis* spp., *Blechnum orientale* L. and *Dicranopteris linearis* L.

ADDITIONAL SPECIMENS EXAMINED.—Sri Lanka. Prov. Sabaragamuwa, Sinharaja forest reserve, 542 m, 19 July 2007, *R.H.G. Ranil*, *78R* (2 sheets), *79R* (4 sheets), *80R* (4 sheets) (PDA); 536 m, 04 July 2008, *R.H.G. Ranil*, *120R* (3 sheets), *121R* (5 sheets), *122R* (3 sheets) (PDA); 540 m, 11 December 2008, *R.H.G. Ranil*, *320R* (3 sheets) (PDA).

DISCUSSION.—In Sri Lanka, *Cyathea srilankensis* remotely resembles *C. hookeri* Thw. from which it is easily distinguished by its larger trunk and leaves as well as by its pinnate to pinnatisect pinnae. *Cyathea srilankensis* cannot be confused with any other tree fern species known from Sri Lanka. From comparisons of morphological characters with known species of the family Cyatheaceae described in Holttum (1963, 1964, 1965a, 1965b, 1974, 1981), Conant (1983), Holttum and Edwards (1983), Large and Braggins (2004), Edwards (2005), Janssen and Rakotondrainibe (2006, 2007, 2008), it was clear that *C. srilankensis* is somewhat morphologically similar only to *C. humilis* Hieron., which occurs in 1,100–2,000 m altitude montane forests of Tanzania and Kenya, and to *C. schliebenii* Reimers occurring in 1,800–2,000 m altitude montane forests in Tanzania. However, *C. srilankensis* can be differentiated from both *C. humilis* and *C. schliebenii* by its longer petioles with sparsely arranged scales, abruptly narrowed leaf base with more sparsely placed and few lower pinnae, absence of hairs from the adaxial face of the rachis and from the abaxial face of the costae (Table 1). This was further confirmed by comparing digital images of type specimens of both *C. humilis* and *C. schliebenii* and specimens available at the Paris herbarium with *C. srilankensis*. According to Holttum (1981) and Edwards (2005), *C. humilis* var. *pyconophylla* differs from *C. srilankensis* by its lamina being 115 cm long, its pinnae being inserted at right angles to rachis, the basal ones 1–1.5 cm long, its largest pinnae being 17 cm long and 2.5 cm wide, its middle pinnae being 2.5 cm apart, and its pinnules being separated by narrow sinuses. No similar species have been reported from southern India, southeast Asia or Australia (Large and Braggins, 2004).

Holttum (1981) has suggested an affinity of Sri Lankan *Cyathea* species with African elements. Such a disjunct pattern might result from an ancestral species that was widespread across the ancient continent of Gondwana (Strahler and Strahler, 2005). It has been shown previously, that Sri Lankan

TABLE 1. Comparison of *Cyathea schliebenii*, *Cyathea humilis* and *Cyathea srilankensis*.

Major characters	*C. schliebenii*	*C. humilis*	*C. srilankensis*
Habitat elevation (m)	1,800–2,000	1,100–2,000	410–665
Trunk height (m)	up to 5	up to 2.5	up to 2.1
Trunk diameter (cm)	up to 3.5	up to 4	up to 5
Petiole length (cm)	10–20	2–10	18–38
Petiole scale arrangement	Dense	Dense	Sparse
Petiole scale size (cm)	1–1.2 × 0.1–0.15	1–1.5 × 0.1–0.5	1–1.2 × 0.1–0.15
Length of lamina (cm)	70–85	40–180	130–202
Width of lamina (cm)	24–30	24–34	40–65
Lower pinnae	Gradually decrescent, pinnae close to each other	Gradually decrescent, pinnae close to each other	Abruptly narrowed, pinnae distant from each other
No. of pinna pairs	20–40	40–50	44–57
Length of lowest pinnae (cm)	4–7	1.5–3	5–7
Length of largest pinnae (cm)	12–15	12–17	20–32
Hairs on rachis (adaxial face)	Present	Present	Absent
Hairs on costa (abaxil face)	Present	Present	Absent
Pinnule margins	Slightly crenate to subentire	Entire, crenate near the apex	Serrate
Veins	Forked, 10–11 pairs per pinnule	Forked, 7–9 pairs per pinnule	Forked, 8–10 pairs per pinnule

tree ferns form a monophyletic group not closely related to African tree ferns (Janssen *et al.*, 2008). However, the phylogenetic position of *C. srilankensis* remains to be elucidated.

ACKNOWLEDGMENTS

The authors are indebted to staff of the National Herbarium of Sri Lanka for their generous support. We are grateful to the curators of the Museum national d'Histoire naturelle Paris and Botanischer Garten und Botanisches Museum Berlin-Dahlem, Germany for providing digital images of *Cyathea humilis* and *Cyathea schliebenii* and scanned images of the type specimen of *Cyathea humilis*, respectively. We also thank Dr. Marcus Lehnert, University of Göttingen, Germany, Dr. Monika Shaffer-Fehre, Kew Herbarium, UK, Mr. Tom Stuart, Coordinator of American Fern Society and Mr. W.P. de Winter, Wageningen University and Research Centre, Netherlands, for providing important information on the family Cyatheaceae. Information provided by Mr. Paris Kamau, National Museum of Kenya, Nairobi and Prof. Chris Fraser-Jenkins, Nepal is also appreciated. We are thankful to Mr. T. M. Rathnayake and Mr. R. Sirimanna for their support during fieldwork. We are also indebted to Mr. T. P. Weeraratne, University of Peradeniya for preparing the line drawings. We thank the Forest Department of Sri Lanka for granting permission to carry out this study at Sinharaja forest and the National Science Foundation of Sri Lanka for financing the research project (RG/06/EB/05).

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American Fern Journal 100(1):45–53 (2010)

Isoëtes laosiensis, a New Species from Lao PDR

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ABSTRACT.—An undescribed species of *Isoëtes* (Isoëtaceae) was found during a floristic survey in Lao PDR. This Laotian species is distinctive enough to identify it as a separate species from the related taxon *I. coromandelina* by its megaspore characteristics, polyhedral shape, large tubercules on proximal and distal surfaces, and the uniform size. We propose *I. laosiensis* as a new species from South East Asia (Laos).

KEY WORDS.—*Isoëtes, Isoëtes laosiensis*, Lao PDR, new species

During the last decade botanical research has become active in Laos (Newman *et al.*, 2007a, b; Thomas *et al.*, 2007). Recently the largest output of floristic research is "A Checklist of the Vascular Plants of Lao PDR" (Newman *et al.*, 2007b), which incorporates data from historical specimen collections in European herbaria as well as more recent botanical field work. However, the distribution of *Isoëtes* species has not yet been reported in this area (Pfeiffer, 1922; Newman *et al.*, 2007a, b). During a recent field survey in Laos, we found an unrecorded *Isoëtes* species similar to *I. coromandelina* L. (*s.l.*) in general appearance.

Isoëtes coromandelina was first described from the Coromandel Coast, Tamil Nadu, India in 1781 by C. Linneaeus, fil. This species has been well documented, with detailed investigations of its mega and microspore morphologies, which have been among the main characters used in delimiting this and other Indian species of *Isoëtes* (Pant and Srivastava, 1962; Singh *et al.*, 1983; Srivastava *et al.*, 1993). Marsden (1976) reported a new subspecies, *I. coromandelina* ssp. *macrotuberculata* Marsden based on the ornamentation of the megaspores (i.e., larger tubercles on the proximal face and irregularly corrugated ridges).

Our comparison between Laotian *Isoëtes* plants and *Isoëtes* species from India and North Australia revealed differences in the ornamentation of spores

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(Pfeiffer, 1922, Pant and Srivastava, 1962; Marsden, 1976; Srivastava *et al.*, 1993; Cook, 1996), and the former are here described as a new species.

Materials and Methods

We collected materials of an unrecorded *Isoëtes* species from Nagang village, Laos (N 14° 46′ 8.8″, E 106° 1′ 44.9″). Voucher specimens are deposited at AJOU. For delimitation of this unrecorded species, we compared it with the original descriptions and herbarium specimens of *I. coromandelina (s.l.)*, *I. indica* Pant & Srivastava, *I. sampathkumaranii* Rao, *I. dixitei* Shende, *I. mahadevensis* Srivastava, Pant, & Shukla, *I. panchganiensis* Srivastava, Pant, & Shukla, and *I. panchananii* Pant & Srivastava from India and North Australia (Table 1 and Appendix 1). As diagnostic morphological characters we examined the number of corm lobes, the number of leaves, leaf length and width, the number of peripheral fiber strands, presence or absence of stomata and velum, shape of labium and ligule, and sporangium length and width (Table 1). Observations, measurements, and photomicrographs of mega and microspores were performed by an Olympus BX40 microscope and a Jeol JSM–6380 scanning electron microscope.

Results and Discussion

Morphology.—The morphological characters for our newly describes species, *I. laosiensis*, and *Isoëtes* species from India and North Australia are listed in Table 1. *Isoëtes laosiensis* differs from Indian *Isoëtes* species, *I. coromandelina*, *I. sampathkumaranii*, *I. mahadevensis*, *I. panchganiensis*, and *I. panchananii*, in the number of corm lobes, presence or absence of peripheral fiber strands in the sporophyll, presence or absence of velum, and shape of labium and ligule. The number of corm lobes has been used in the taxonomy of *Isoëtes* (Pfeiffer, 1922; Takamiya *et al.*, 1997). *Isoëtes laosiensis* has a three lobed corm, whereas *I. mahadevensis* and *I. panchananii* typically have two lobed corms (Pant and Srivastava, 1962; Srivastava *et al.*, 1993). The peripheral fiber strand is a mechanical tissue which consists of a few short fibers and situated under the epidermis in the sporophyll (Takamiya *et al.*, 1997). *Isoëtes laosiensis* has them at four sites, whereas no such strand is found in *I. sampathkumaranii*, *I. mahadevensis*, and *I. panchananii* (Pant and Srivastava, 1962; Srivastava *et al.*, 1993). A velum is absent in *I. laosiensis* but those of *I. sampathkumaranii*, *I. panchganiensis*, and *I. panchananii* are more or less well developed covering about half to all of the surface of the sporangium (Pant and Srivastava, 1962; Srivastava *et al.*, 1993). The labium of *I. laosiensis* is absent or very short, extending to 1/5 of the total ligule length, whereas the labium of *I. coromandelina (s.l.)* is conspicuous, hemiorbicular, covering all but the apex of the ligule (Table 1). The ligules of *I. laosiensis* and *I. coromandelina* were similar, being elongated deltoid in shape (Fig. 1C), whereas those of *I. indica*, *I. dixitei*, *I. mahadevensis*, *I. panchganiensis* and *I. panchananii* have been described as triangular or deltoid with a cordate base (Table 1).

The measurements of morphological characters from our new species, including the number of leaves, leaf length and width, presence or absence of stomata, and sporangium length and width are consistent with those previously observed in *Isoëtes* species from India and North Australia (Pfeiffer, 1922, Pant and Srivastava, 1962; Marsden, 1976; Cook, 1996). Therefore, it is difficult to distinguish species by many morphological characters. In many cases, vegetative characters of *Isoëtes* are considered of limited taxonomic value because of their infraspecific variability or their interspecific uniformity (Hickey, 1986a). It has been also well documented that many vegetative characters vary as the result of phenotypic plasticity (Johnson, 1984; Cook, 1996; Britton *et al.*, 1999). This could be why we did not determine the key characters for delimitation of *I. laosiensis* based on its external appearance.

Spore ornamentation and size.—Identification of *Isoëtes* species largely rests on megaspore and microspore ornamentation and spore size (Pfeiffer, 1922; Hickey, 1986b; Macluf *et al.*, 2006; Choi *et al.*, 2008). Megaspores of *I. laosiensis* have a tuberculate ornamentation on both the proximal and distal surfaces (Fig. 2A–D). Based on the figures in Pant and Srivastava (1962) and Srivastava *et al.* (1993), there are four quillworts in India with tuberculate megaspores: *I. coromandelina*, *I. indica*, *I. sampathkumaranii*, and *I. dixitei* (Table 1). The tubercules in the megaspore of *I. laosiensis* are mostly rounded, while those in the megaspores of *I. indica* are gradually tapering towards their pointed ends (Pant and Srivastava, 1962). Moreover, the commissural ridges in megaspores of *I. laosiensis* are straight and smooth, while they are sinuous in *I. indica* (Table 1). The surface of megaspores in *I. sampathkumaranii* and *I. dixitei* shows a number of uneven tubercles both on the proximal and the distal sides, but a few or a single tubercle is seen in each pyramic area on the proximal side of *I. laosiensis* (Table 1 and Fig. 2B). Megaspore ornamentation of *I. laosiensis* is similar to that of *I. coromandelina*. However, megaspore ornamentation of *I. laosiensis* differs from those of *I. coromandelina* ssp. *coromandelina* in having markedly larger tubercles on both the proximal and distal surfaces (Fig. 2A–H). Although it is similar to *I. coromandelina* ssp. *macrotuberculata*, it differs because of its triradiate and commissural ridges, with *I. laosiensis* being straight and *I. coromandelina* ssp. *macrotuberculata*, irregularly corrugate (Table 1). Moreover, megaspores of *I. laosiensis* are uniform in size (595.0 ± 36.9 μm), whereas megaspores of *I. coromandelina (s.l.)* are always distinctly dimorphic in a single sporangium (Pant and Srivastava, 1962; Marsden, 1976) (Table 1 and Fig. 2A, E).

Microspores ornamentation of *I. laosiensis* is a laevigate on proximal surface and echinate on the distal faces (Fig. 2I–L). In texture, the microspores of *I. laosiensis* are similar to *I. coromandelina* (*s.l.*). However, *I. laosiensis* microspores (34.6 ± 2.2 μm) are larger than *I. coromandelina* (26–33 μm), although there is some overlap in the size ranges (Table 1).

Our spore morphological data indicate that *I. laosiensis* is distinctive for identification as separated species from *I. coromandelina* (*s.l.*) and the other *Isoëtes* species in India and North Australia (Table 1). The following is description of previously unnamed species of *Isoëtes*:

TABLE 1. Comparison the morphological characters of *I. laosiensis* with the previous descriptions of *Isoëtes* in India and North Australia.

Morphological characters	*laosiensis*	*coromandelina* ssp. *coromandelina*[a, b]	*coromandelina* ssp. *macrotuberculata*[b]	*indica*[a]
No. of corm lobes	3	3 (rarely 4, 5)	3 (rarely 4, 5)	3 (rarely 4)
No. of leaves	21 – 42	20 – 60	15 – 60	9 – 35
Leaf length (cm)	20.6 ± 3.8	< 60	60 – 80	8 – 55
Leaf width (mm)	2.1 ± 0.5	ND	ND	ND
Peripheral fiber strand	4	4	4	4 – 6
Stomata	present	present	present	present
Velum	absent	absent	absent	absent
Labium	absent or very short	conspicuous, hemiorbicular	conspicuous, hemiorbicular	ND
Ligule shape	elongated deltoid	elongated deltoid	lanceolate	deltoid with cordate base
Sporangium length (mm)	5.4 ± 1.2	< 12	< 12	6 – 19
Sporangium width (mm)	3.5 ± 0.4	< 9	< 7	4 – 9
Megaspore ornamentation	tuberculate (globular large tubercles)	tuberculate (numerous globular small tubercles)	tuberculate (large and small globular tubercles)	tuberculate (tubercles tapering mostly with polar end)
Megaspore diameter (um)	595.0 ± 36.9	small (356 – 458) large (465 – 660)	small (330 – 410) large (420 – 530)	small (89 – 380) medium (407 – 509) large (458 – 636)
Megaspore ridges	straight and smooth	straight and smooth	irregularly corrugate	sinuous
Microspore ornamentation	smooth with spines	smooth or rugulose to papillate or spines	ND	finely tuberculate
Microspore length (um)	34.6 ± 2.2	26–33	ND	16 – 48

Isoëtes laosiensis C. Kim & H.–K. Choi, ***sp. nov.***—TYPE: Laos, Ban Kiat Nagang village, Mt. Phou Asha (N 14° 46′ 8.8″, E 106° 1′ 44.9″), elev. 150–300 m, 24 July 2007, B.Y. Sun and S.S. Choi 2039–5 (Holotype, AJOU; Isotype, AJOU). **Figs. 1 and 2.**

Megasporae triangularis in proximalis aspectus, monomorphicae, 533.9–(595.0)–697.8 μm diam., hemisphaerium proximalis et distalis major tuberculares, juga triradiata et commissuralia laevis.

Plant amphibious. Corm globose, 3–lobed with abundant dichotomous roots. Sporophylls white basally, green above, ascending, 9.7–(20.6)–27.0 cm long,

TABLE 1. Extended.

dixitei[a]	*sampathkumaranii*[a]	*mahadevensis*[c]	*panchganiensis*[c]	*panchananii*[a]
3	ND	2 (rarely 3)	3	2
ND	ND	5 – 29	9 – 20	4 – 38
ND	ND	< 14	7 – 13	7 – 24
ND	ND	1.0 – 1.5	1.2 – 3.0	ND
present	Absent	absent	ND	absent
ND	ND	present	present	present
rudimentary (rarely covering about half of the sporangium)	present (covering about half to 2/3 of sporangium)	rudimentary	present (covering nearly the entire sporangium)	present (covering about half of sporangium)
ND	ND	triangular with obtuse apex	triangular	ND
deltoid with cordate base	ND	triangular with lobbed base	triangular with expended base	triangular
ND	ND	3 – 4.5	3 – 4	3 – 5
ND	ND	2 –2.5	2.5 – 3	2 – 3
tuberculate (uneven tubercles)	tuberculate–cristate	rugulose or pustule	reticulate	reticulate
small (320 – 458) large (483 – 660)	small (280 – 381) large (356 – 458)	small (203 –372) large (384 – 507)	small (185 – 305) large (270 – 456)	small (240 – 330) large (330 – 407)
straight	straight or slightly sinuous	undulating	straight or sinuous	straight or slightly sinuous
ND	ND	echinate	ND	ND
ND	ND	10 – 47	ND	ND

ND, not determined by original authors.
[a]Pant and Srivastava (1962);
[b]Marsden (1976);
[c]Srivastava *et al.* (1993).

1.0–(2.1)–3.0 mm wide at mid–length, in tufits of 21 to 40, half–terete, base alate. Peripheral fiber strands present. Stomata anomocytic, 67.2–(73.4)–79.1 μm long, 25.2–(35.7)–51.1 μm wide, 6–8 peristomatic neighbouring cells. Velum absent. Labium absent or very short, deltoid segment, broad at base. Ligule membranaceous, elongated deltoid. Sporangia orbicular to ovovate, wall pale to brownish, 3.3–(5.4)–8.2 mm long, 2.6–(3.5)–4.3 mm wide.

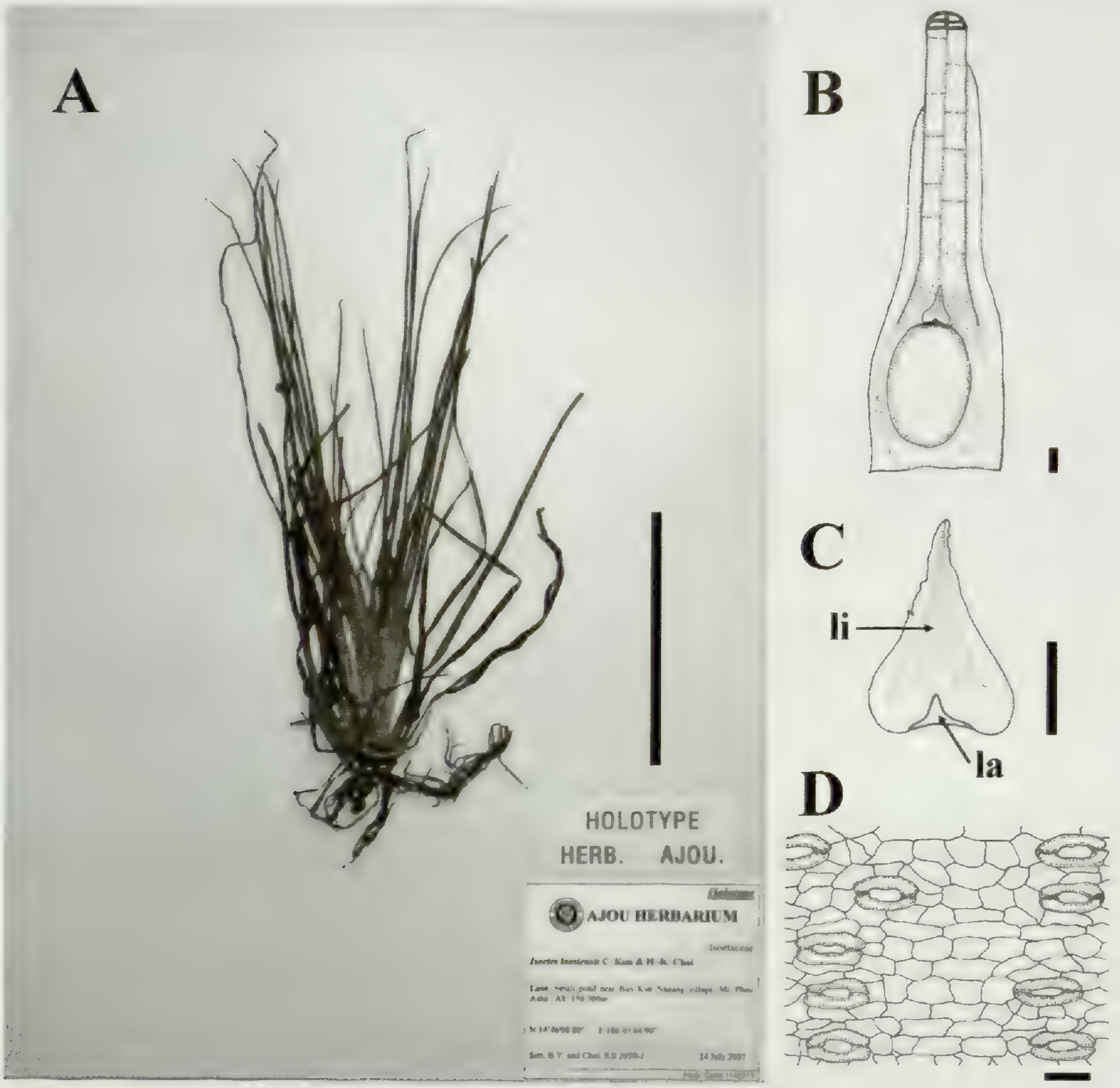

FIG. 1. *Isoëtes laosiensis* C. Kim & H.–K. Choi, *sp. nov.* **A.** Habit of plant (Nagang villiage, Laos, 24 July 2007, Sun and Choi *2039–1* [Holotype]). **B.** Adaxial view of basal portion of sporophyll. **C.** Shape of labium and ligule. la, labium; li, ligule. **D.** Stomata. Scale bars: A = 10 cm; B and C = 1 mm; D = 50 μm.

Megaspores triangular in proximal view, large tubercules on both the proximal and distal surfaces, white in dry, grey in wet, 533.9–(595.0)–697.8 μm diameter, 399.5–(480.0)–671.2 μm height, triradiate and commissural ridges straight and smooth, ultrastructure fibrous. Microspore brown, 29.2–(34.6)–40.4 μm long, 20.5–(26.0)–34.3 μm diameter, proximal hemisphere laevigate, distal hemisphere echinate, ultrastructure rugulae and granules.

ETYMOLOGY.—This species epithet *laosiensis* is derived from the name for Laos, on which the new species has been collected.

DISTRIBUTION.—This species is known only from Laos, but will be probably be found to occur widely than now recorded. At present, *Isoëtes* is poorly known through South East Asia, possibly in part because of difficulty in field recognition of the genus.

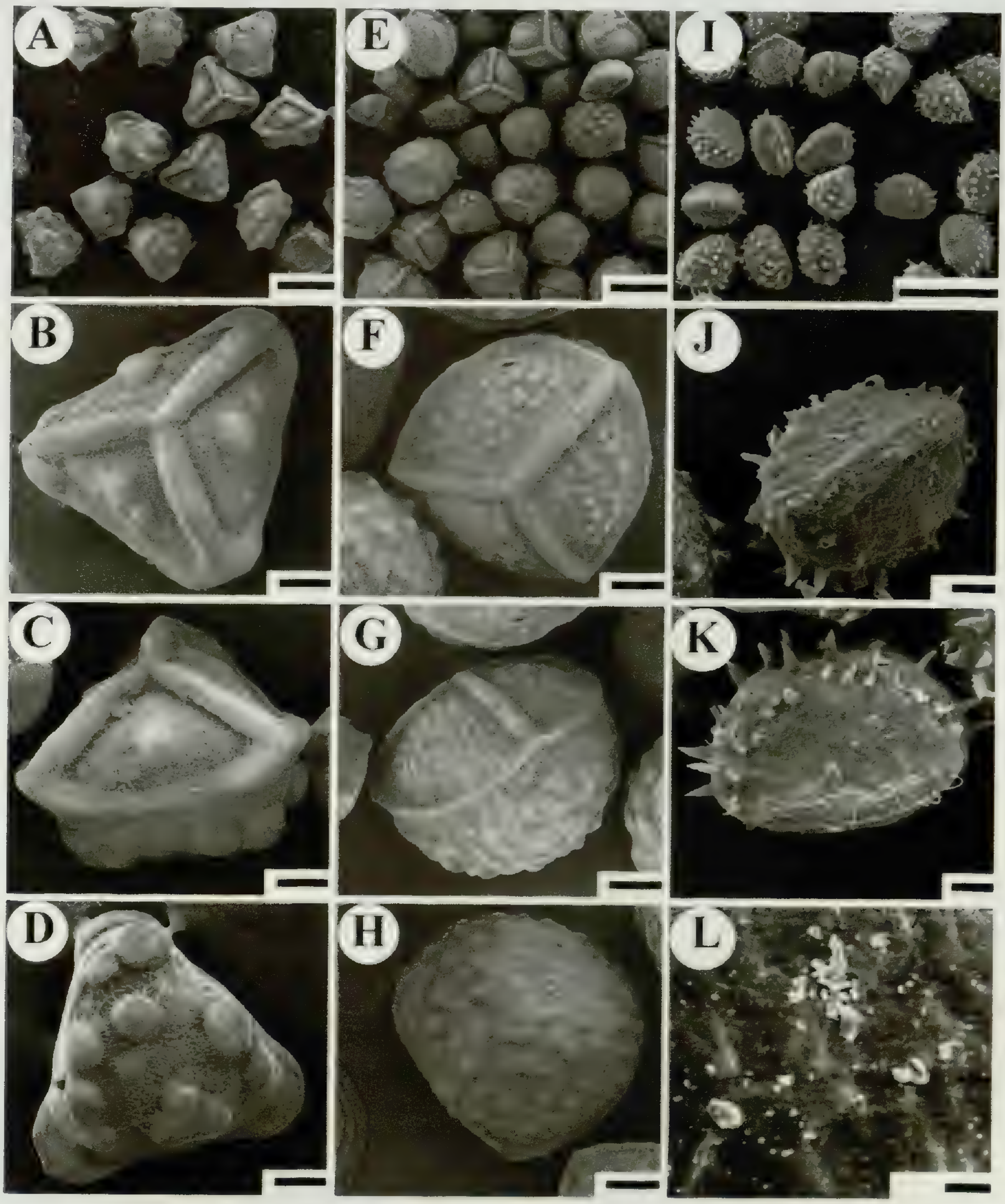

FIG. 2. Spore morphology of *I. laosiensis* C. Kim & H.–K. Choi, sp. nov. and *I. coromandelina* L. fil. **A–D.** Megaspores of *I. laosiensis* (Sun and Choi *2039–5* [Isotype]). **A.** Overview. **B.** Proximal view. **C.** Lateral view. **D.** Distal view. **E–H.** Megaspores of *I. coromandelina* (India. Rajasthan, Sept 29 1995, C.D.K. Cook *5327* [K]). **E.** Overview. **F.** Proximal view. **G.** Lateral view. **H.** Distal view. **I–L.** Microspores of *I. laosiensis* (Sun and Choi *2039–5* [Isotype]). **I.** Overview. **J.** Proximal view. **K.** Lateral view. **L.** Ultrastructure of microspore surface. Scale bars: A and E = 500 μm; B–D and F–H = 100 μm; I = 50 μm; J–K = 5 μm; L = 1 μm.

Acknowledgments

The authors gratefully acknowledge the curators and directors of the herbaria K and E, for providing access to herbarium specimens. We also thank to two anonymous reviewers for comments on the manuscript. This work was supported in part by grants from the Ministry of Environment as parts of the Eco–Technopia 21 project (KIEST 052-071-049) to HKC and by International Cooperation Unit of Biodiversity and Environmental Conservation (ICUBEC) to BYS.

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Appendix 1. Herbarium specimens examined in this study. Herbaria abbreviations: Royal Botanic Garden, Kew (K) and Royal Botanic Garden, Edinburgh (E).

***Isoëtes coromandelina* L. fil. ssp. *coromandelina*—India.** Kunigal, Sept 16 1969, D.A. Govindappa *120* (K); Mysore, Oct 27 1970, F.M. Jarrett *592* (K); same locality, Nov 26 1971, S.S. Hooper & C. Saldanha *s.n.* (K); Kerala, Dec 16 1985, V.S. Manickam & K.M. Matthew *s.n.* (K); same locality, Dec 21 1985, V.S.

Manickam and K.M. Matthew *s.n.* (K); Rajasthan, Sept 29 1995, C.D.K. Cook *5327* (K); Meerut, unknown date, S.N. Bhambie *1955* (K). **Sri Lanka.** Uva Province, Dec 26 1976, R.B. & A.J. Fadan *76/603* (K); Northern Provinve, Jan 13 1977, R.B. & A.J. Fadan *77/160*, *77/236* (K); Ceylon, Dec 20 1881, C. Trimen *7/82* (K).

***I. coromandelina* ssp. *macrotuberculata* Marsden—Australia.** Northern Territory, May 22 1983, P.A. Fryxell & L.A. Craven *4249* (E).

***I. dixitei* Shende—India.** Mysore, Oct 15 1969, K.B. Sadanaudra & S.N. Ramaswamy *204* (K); same locality, Aug 18 1973, S.S. Patil *2* (K).

***I. indica* Pant & Srivastava—India.** Madhya Pradesh, Nov 13 1960, D.D. Pant *2G* (K).

***I. panchananii* Pant & Srivastava—India.** Madhya Pradesh, unknown date, D.D. Pant *1* (K); same locality, Nov 13 1960, D.D. Pant *1A*, *1G* (K).

***I. sampathkumaranii* Rao—India.** Chikkamagalur, May 12 1968, S.N. Ramasaramy *118* (K).

American Fern Journal 100(1):54–69 (2010)

Shorter Notes

***Asplenium abscissum* Willd. (Cutleaf Spleenwort) in Alabama.**—*Asplenium abscissum* Willd. (Cutleaf Spleenwort) is a tufted fern with unbranched, brown-scaled stems, leaves that are deltoid in outline with lanceolate pinnae and a fibrous root system (Nelson, *The Ferns of Florida: A Reference and Field Guide*. Pineapple Press, Inc. Sarasota, FL. 2000). Nelson also reports that spores mature year-round from sori that arise in pairs along the edge of lateral veins. This lithophilic fern is almost always associated with some form of limestone, as in eroded rockland hammocks, cliff ledges, cave entrances, or watery grottoes (FNA, Flora of North America. Editorial Committee, eds. 1993+. *Flora of North America North of Mexico*. New York and Oxford. Vol. 2, pg. 236. 1993). *Asplenium abscissum* is considered a New World tropical species that is distributed from Brazil south to Bolivia, throughout Central America and Mexico, the West Indies of the Antilles, and thinly spread across seven southern/central Florida counties in the continental U.S. (FNA, 1993; Kartesz, Floristic Synthesis of North America. BONAP. *In Press*. 2010).

This paper reports the recent discovery and documentation of a new Cutleaf Spleenwort population for Jackson County, Alabama, 6.5 km (4 mi) south of the Tennessee border at an elevation of 925 ft (282 m) above sea level (Fig. 1). The next closest known population occurs approximately 800 km (500 mi) to the southeast in Alachua County, Florida (near Gainesville). While historically much of lower Alabama was covered with a shallow sea and the climate was more tropical in nature (Lacefield, *Lost Worlds in Alabama Rocks: A Guide to the State's Ancient Life and Landscapes*. Alabama Geological Society. Tuscaloosa, AL. 2000), today Jackson County, Alabama lies at the extreme northern fringe of what is considered subtropical climate.

At the Alabama site, approximately 100 fronds from 50 sporophytes in many stages of development were counted (Fig. 2). Numerous gametophytes were also observed growing along the same 2 m^2 stretch of the east/southeast facing vertical flowstone wall of the cave entrance. *Adiantum capillus-veneris* L. (Venus' Hair Fern) and many bryophytes were also present. The Cutleaf Spleenwort was found growing 6 m below the surface entrance to the vertical cave, with light levels becoming greatly diminished below the fern habitat (approximately 8 m from the surface). Environmental conditions of the cave are modified by a restricted entrance, the "chimney effect" which keeps the ambient temperature from falling below 14°C (56°F) near the plants at any point during the year, and a perennial cave stream approximately 18 m below the fern habitat providing an ambient humidity that is elevated. However, condensation forming on the cave wall and rainfall appear to be the only water sources for the ferns since the flowing water is well below the plants.

Jackson County, the most northeastern county in Alabama, contains the highest number of caves in the state, with this network of caves hosting among the highest number of troglodytic (cave-dwelling) and the highest number of

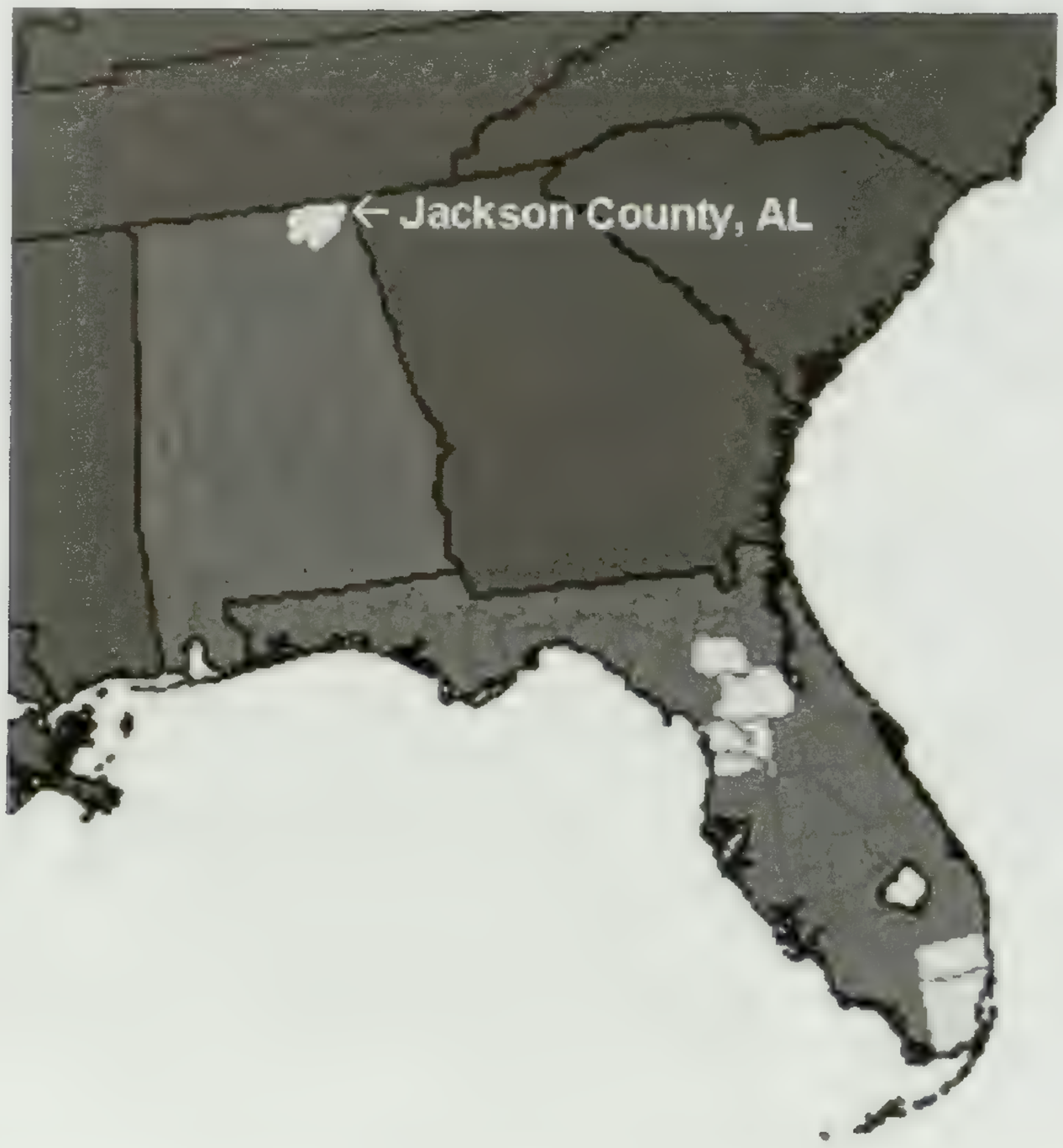

FIG. 1. Highlighted locations of the eight known county records for *Asplenium abscissum* in the continental U.S.

FIG. 2. Image of the vertical cave habitat and growth habit of *Asplenium abscissum* in Jackson County, AL. The mature fronds pictured are approximately 15–20 cm in length.

troglobitic (cave dwelling animals adapted to complete darkness) species in the U.S. (Kidd, Encyclopedia of Alabama [online edition]. http://www.encyclopediaofalabama.org/face/Article.jsp?id=h-20202009. 2009). Although often less celebrated than animal species, many plant species have also become specialists of cave habitats. These populations are often times disjunct from core populations. The processes creating such isolated populations were described by Small in his 1938 book, *Ferns of the Southeastern States*:

> "Many of the sinks are evidently the result of the roofs of these caverns falling in. The sinks have become ferneries, but their species are largely the same. Where a small portion of a roof has fallen in leaving a well-like opening, unusual ferns are to be expected where anchorages are available on the walls..."

Also noted by Small (*Ferns of the Southeastern States*. The Sciences Press Printing Company. Lancaster, PA. 1938), floral components are often dramatically different when associated with unusual geological features. Cave entrances can provide habitats approaching more tropical conditions with respect to humidity and temperature. The physical conditions provided by such unusual features may be ideal for species that are considered specialists of a particular niche. Habitat comparisons for the Alabama and Florida populations of Cutleaf Spleenwort illustrate this fact. The Florida populations are typically found growing on limestone substrates surrounded by mixed tropical hardwoods, frequently interspersed with *Quercus virginiana* Mill. (Live Oak) (Nelson, 2000). The Alabama population is surrounded by an ecotone with many *Quercus* and *Carya* species in a dry oak/hickory forest with limestone outcroppings.

Although not listed by the U.S. Fish and Wildlife Service, NatureServe (NatureServe, NatureServe Explorer: An online encyclopedia of life [web application]. Version 6.1. NatureServe, Arlington, VA. Available online at http://www.natureserve.org/explorer. Accessed September 1, 2009) has designated the Cutleaf Spleenwort as a G3 species indicating that it is globally vulnerable to extirpation or extinction. In Florida, *A. abscissum* has an S2 ranking (NatureServe, 2009). With the sole known Alabama population occurring in such a fragile habitat, the fern should obtain an immediate S1 ranking in Alabama. Small (1938) mentions that *A. abscissum* has been an inhabitant of Florida "for a very long time" and describes discrete events that likely led to the separated populations in Florida (Fig. 1). One event that he described alludes to the tiny fern spores being dispersed for great distances by the wind. In fact, many of the tropical ferns that grow in Florida are now believed to have arrived there from tropical America or the West Indies via hurricanes and other storms (Spurr, Amer. Fern J. 31: 91–97. 1941). Over the course of millennia, spores could have arrived in Jackson County, Alabama by the same means. While it is impossible to say exactly what events led to the establishment of *A. abscissum* in this Jackson County vertical cave, one can speculate that it is simply a relict population from a time when the climate in this part of Alabama was more tropical in nature.

Voucher: USA. Alabama. Jackson County: Near Stevenson. Three miles south of Russell Cave National Monument and 2.5 mi northwest of U.S. Highway 72. Approximately 100 mature fronds and 50 individuals were growing on a wet flowstone wall of a vertical cave approximately 6 m below the entrance. The overall depth of the cave reaches nearly 60 m with the surface entrance located 925 ft (282 m) above sea level. GPS coordinates withheld. July 10, 2009. *Barger* #SP107 (AUA, JSU, TROY).

The authors thank Curtis Hansen and Jack Short along with the anonymous reviewers for their helpful comments in crafting this manuscript. Special thanks are extended to Dan Spaulding for his expert assistance with plant specimen identification and to Misako Nishino and John Kartesz for verification of county record data and the distribution map.—T. Wayne Barger, Natural Heritage Section, Alabama Department of Conservation, 64 N. Union Street, Montgomery, AL, 36130 U.S.A., Alan Cressler, U.S. Geological Survey, 3039 Amwiler Road, Suite 130, Peachtree Business Center, Atlanta, GA, 30360 U.S.A., Brian D. Holt, Natural Heritage Section, Alabama Department of Conservation, 64 N. Union Street, Montgomery, AL, 36130 U.S.A., and Max Medley, 519 W. Hawthorne A-13, Dalton, GA 30720 U.S.A.

Review of Two Non-Native, Invasive Climbing Ferns (*Lygodium japonicum* and *L. microphyllum*), Sympatric Records and Additional Distribution Records from Florida.—Two non-native species of climbing fern, *Lygodium japonicum* (Thunb.) Swartz and *L. microphyllum* (Cav.) R. Br. are currently present in Florida and are highly invasive in many natural areas of Florida (Gordon, Ecol. Appl. 8:975–989. 1998; Langeland *et al.*, *Identification and Biology of Nonnative Plants in Florida's Natural Areas* (2nd edition). IFAS Communication Services, University of Florida, Gainesville, FL. 2008). Both of these non-native ferns have become problematic to land mangers in natural areas of Florida (Hutchinson *et al.*, Old World Climbing Fern (*Lygodium microphyllum*) Management Plan for Florida. South Florida Water Management District, West Palm Beach, FL. http:// www.fleppc.org. 2006; FLEPPC, Florida Exotic Plant Pest Council's 2007 Invasive Plant Species List. Available on at: http://www.fleppc.org/list/07list.htm. 2007). Both ferns exhibit indeterminate growth growing horizontally and vertically, often forming complete cover over native vegetation. From 2005–2006, the total costs associated with control of upland invasive plants on Florida public lands (cities, counties, state and federal) was 9.4 million dollars (FLDEP, Florida Department of Environmental Protection, Bureau of Upland Plant Management (http://www.dep.state.fl.us/lands/invaspec/2ndlevpgs/Uplandsplntman.htm). 2009). The Florida Fish and Wildlife Conservation Commission's Bureau of Invasive Plant Management currently ranks *Lygodium microphyllum* (Cav.) R. Br. along with hydrilla [*Hydrilla verticillata* (L.f.) Royal] as its priorities for research funding on control methods (Schmitz, Nat. Areas J. 27:251–253. 2007). In 2008,

L. microphyllum and L. flexuosum (L.) Sw. were recommended to be listed as Federal Noxious Weeds (USDA, *Weed Risk Assessment*. United States Department of Agriculture, Animal and Plant Health Inspection Service, Raleigh, N. C. 2008). While *L. flexuosum* is not currently present in the U.S.A., it has similar biological traits (i.e., long distance dispersal, ability to self-cross, fire tolerance) as both species of *Lygodium* now present in Florida (Lott *et al.*, Am. J. Bot. 90:1144–1152. 2003) and may be considered for ornamental use (USDA, 2008). The goals of this study were to examine the historical records of both climbing ferns currently present in Florida based on herbarium records, report on new sympatric records, and document new distribution records from north and south Florida.

The earliest known herbarium record of *L. microphyllum* in Florida is from Davenport in Polk County from January 6, 1958 (Florida Division of Plant Industry, Record # 0-3753). This report was for a specimen at Hollybrook Nursery indicating the fern was present in Polk County at this time. The specimen was turned in because of a fungus on the upper surface of the pinnae. This may indicate that the nursery was cultivating the fern if there was concern about the fungus or that they discovered a plant with a disease and wanted identification. The report suggests using Dithane Z-78 to control the fungus but no other details exists in the report. The second earliest herbarium record for *L. microphyllum* was reported February 11, 1958 (FLAS #P5100) from a nursery in Delray Beach, Florida on the southeast coast. There was no more information on this sample, but the plant was growing in a nursery indicating that it was being propagated or possibly arrived accidentally with other plants. These two reports indicate that *L. microphyllum* was present in 1958 at two different locations in Florida that are ca. 240 km apart. Old World climbing fern has spread across the landscape of Florida at a rapid rate since that time (reviewed in Pemberton and Ferriter, Amer. Fern J. 88:165–175. 1998). This species has been present in Florida for > 50 years and was first documented as naturalized in southeast Florida in 1965 (Beckner, Amer. Fern J. 58:93–94. 1968).

Based on herbarium records, *L. japonicum* has been present in Florida for at least 77 years. The earliest herbarium record of *L. japonicum* was from Polk County in 1931 (USF Accession #175179). It is widely scattered throughout Florida from the panhandle to extreme south Florida. The earliest herbarium records from the panhandle region of Florida are from 1948–1950. This species occurs throughout the southeast United States and has likely been introduced into Florida multiple times. Except for herbarium records, little information exists on *L. japonicum* in Florida. In northern Florida, it was reported that pine straw used for mulch contained *L. japonicum* spores and this has increased the dispersal and spread of the fern (Zeller and Leslie, Wildland Weeds. Summer: 6–9. 2004). It has been reported to form dense cover over native ground and shrub plants in both disturbed sites and natural areas of northern Florida (Langeland *et al.*, 2008).

Based on herbarium records, Polk and Highlands Counties in south-central Florida were the first known sites of both *L. japonicum* and *L. microphyllum* in

Florida. A 1935 record (FLAS P3270) exists for *L. japonicum* from the Florida Botanical Garden and Arboretum in Highlands County which later became part of Highlands State Park. In 1973, a park service biologist documented *L. microphyllum* as naturalized in Highland Hammock State Park (FLAS P7718). In a 2004 aerial surveys of Highlands and Polk Counties along the Lake Wales Region of south-central Florida, 213 locations with *Lygodium* spp. were documented with some infestations of *L. microphyllum* as large as 40–80 ha (Biehl, Wildland Weeds 7(3):4–5. 2004).

In a survey of Florida flora from 1927–1930, no reports of *Lygodium* spp. were documented (Moldenke, Am. Midl. Nat. 32:529–590. 1944). Since the 1930's, two non-native species of climbing fern, *L. japonicum* and *L. microphyllum*, have been documented from herbarium records in Florida (FLAS, University of Florida Herbarium (http://www.flmnh.ufl.edu/herbarium/). Gainesville, Florida. 2008; FSU, Florida State University Herbarium (http://herbarium.bio.fsu.edu/index.php). Tallahassee, Florida. 2008; FTG, Fairchild Tropical Gardens Herbarium (http://www.virtualherbarium.org/default.html). Miami, Florida. 2008; Wunderlin and Hansen, Atlas of Florida Vascular Plants (http://www.plantatlas.usf.edu/). Institute for Systematic Botany, University of South Florida, Tampa. 2008). Both species are listed as Category I invasive species by the Florida Exotic Pest Plant Council indicating they are disrupting natural communities by displacing native plant species and interrupting natural processes (FLEPPC, 2007). The Florida Department of Agriculture and Consumer Services prohibit the sale, propagation, or possession of both *L. japonicum* and *L. microphyllum* in Florida (FDACS, 2009). There is some documentation of these two non-native ferns occurring within the same habitat in close proximity. Prior to May 2000, both *L. japonicum* and *L. microphyllum* were documented in Little Manatee River State Park (Myers and Wunderlin, Castanea 68:56–74. 2003) indicating both species were established in west central Florida prior to 2000. Lockhart (Statewide *Lygodium* treatment site evaluation project. Florida Natural Areas Inventory, Tallahassee, FL. Available on at: http://www.fnai.org/pdf/Lygodium_Report_2007.pdf. 2007) reported that both *L. japonicum* and *L. microphyllum* occurred at Avon Park Bombing Range and along the Kissimmee River Floodplain in Polk County. Both species were also observed growing together along a disturbed mesic / hydric ecotone in Sarasota and Palm Beach Counties in south Florida (Lockhart, 2007).

Nauman and Austin (Amer. Fern J. 68:65–66. 1978) reported *L. japonicum* and *L. microphyllum* were both well-established in different parts of Florida and suggested the two species would meet in central Florida. Historically, *L. japonicum* was most common north of Orlando with records as far south as Collier County, while *L. microphyllum* was most common south of the northern section of Lake Okeechobee with scattered populations into Polk County to the north (Pemberton and Ferriter, 1998). Herbarium records accessed on the internet from the University of Florida (FLAS, 2008), Florida State University (FSU, 2008), University of South Florida (Wunderlin and Hansen, 2008) and Fairchild Tropical Gardens (FTG, 2008) dating from 1930–2008 indicate that 70% (n = 190) of documented occurrences of *L. japonicum* occur north of

TABLE 1. The five earliest known herbarium records for *L. japonicum* and *L. microphyllum* in Florida based on herbarium records.

Date	Accession #	County	Location
		L. japonicum	
March, 1931	USF 175179	Polk	Winter Haven
March, 1932	FLAS P5939	Dade	Miami (vacant lot)
Unknown, 1934	FLAS P207	Alachua	Gainesville (cultivated)
January, 1935	FLAS P3270	Highlands	Sebring
January, 1935	FLAS P208	Collier	Naples (cultivated)
		L. microphyllum	
January, 1958	DIP 0-3753	Polk	Davenport (nursery)
February, 1958	FLAS P5100	Palm Beach	Delray Beach (nursery)
August, 1960	FSU 4658	Martin	Jupiter
June, 1965	FLAS P6659	Martin	Hobe Sound
November, 1966	FLAS P6980	Martin	Jupiter

Orlando (N 28° 25′–WGS 84 Datum) while 98% (n = 91) of documented occurrences of *L. microphyllum* occur south of Orlando. Pemberton (Sida 20:1761–1761. 2003) reported a new population of *L. microphyllum* in east Orlando that increased the northern distribution by 70 km, an indication that the two species ranges have over-lapped further north than reported by Myers and Wunderlin (2003). A 2005 herbarium record from Volusia County, Florida indicated that *L. microphyllum* occurred ca. 90 km north of Orlando (USF #540261). This population of *L. microphyllum* was located ca. 3.5 km NE of Ormond Beach and ca. 13.0 km north of Daytona Beach.

Of the five earliest known herbarium records of *L. japonicum* in Florida, four of these records are from locations south of Orlando, two of which are from the southern end of the peninsula in Dade and Collier County (Table 1). However, *L. japonicum* has not spread across the landscape of south and central Florida to a similar extent as *L. microphyllum* and is more invasive in north Florida. Twenty-one percent of the herbarium records for *L. japonicum* that occur south of Orlando are from areas in Hillsborough, Pinellas, Manatee and Polk Counties indicating that *L. japonicum* has long been established in west-central Florida. Only 3.7% (n = 7) of documented records for *L. japonicum* occur in Dade and Collier Counties in the southern peninsula of Florida.

In 2005, the combined coverage of both species of *Lygodium* based on aerial surveys was estimated to be 74,122 ha in the central and south regions of Florida, with 48,898 ha in the southern region, most of which was likely *L. microphyllum* (Ferriter and Pernas, Wildland Weeds 9(2):7–9. 2006). During floral surveys of Water Conservation Areas 2 and 3 in the central Everglades, no reports of *Lygodium* spp. were documented in the late 1950's (Loveless, Ecology 40:1–9. 1959), but presently *L. microphyllum* is common on many tree islands in the central and northern Everglades. There are no known documented reports on the area coverage of *L. japonicum* and its area coverage in the central and northern Florida regions is unclear. Based on GIS analysis (Google Earth, http://earth.google.com. 2008) of *Lygodium* spp. in Florida from documented and undocu-

TABLE 2. City, county, GPS coordinates, year observed and habitat type of locations where *Lygodium japonicum* and *L. microphyllum* occurred concurrently in Florida.

City	County	GPS Coordinates[1]	Year Observed	Habitat Type
Lakeland	Lake	N 27° 55′ 39.6″, W 081° 57′ 06.8″	2006	Maple swamp
Clermont	Lake	N 28° 23′ 37.3″, W 081° 45′ 51.8″	2008	Cypress strand
Naples	Collier	N 26° 12′ 47.7″, W 081° 45′ 52.2″	2008	Cypress strand
Balm	Hillsborough	N 27° 44′ 26.4″, W 082° 17′ 43.6″	2006	Maple swamp

[1]- WGS 84 Datum

mented records, *L. microphyllum* currently ranges ca. 430 km in latitude (north to south) in peninsula Florida, while *L. japonicum* ranges ca. 550 km in longitude (east to west) along the panhandle and northern Florida, and ca. 570 km in latitude (north to south) along the peninsula. Based on herbarium records, this range expansion of both *Lygodium* spp. in Florida has occurred in < 80 years.

Expansion of *L. japonicum* is likely due to cultivation and propagule dispersal throughout the state from multiple introductions. The expansion of *L. microphyllum* is likely due to accidental introduction. The earliest known herbarium records of *L. japonicum* indicate it was first known from cultivation, while the earliest records of *L. microphyllum* indicate it was present in two nurseries. It has been reported that *L. microphyllum* was used as an ornamental (Gordon and Thomas, *in* D. Simberloff, D. C. Schmitz and T. C. Brown, *Strangers in Paradise*. Island Press, Washington, D. C. 1997). The introductions of these two climbing ferns, as well as many other non-native plants, indicates the need for better screening and evaluation of new plant introductions into Florida (Gordon, 1998; Gordon *et al.*, Invas. Plant Sci. Mange. 1:178–195. 2008).

New sympatric records.—From 2006–08, we observed four locations in which both *Lygodium* species occurred concurrently (Table 2). At three sites (Lakeland, Clermont, and Balm), there was a distinct habitat delineation between the two species. *Lygodium japonicum* was present at slightly higher elevations in a soil mixture of sand/humus, while *L. microphyllum* was present at lower elevations in humus soils. Elevation differences at sites where *L. japonicum* and *L. microphyllum* occurred together were < 1.0 m. Soils where *L. japonicum* was present were more xeric to mesic, while soils where *L. microphyllum* were present were more mesic to hydric. In addition, *L. japonicum* was observed in more open sites that were exposed to sunlight while *L. microphyllum* was observed in more shaded sites with > 50% canopy cover. At the Naples site, we observed both species growing together in a cypress strand, with *L. microphyllum* accounting for >99% cover. Canopy cover at the Naples site was >75% indicating that sunlight may be a factor in the distribution of these two ferns in Florida.

At Archbold Biological Station in Highlands County, one author (JTH) observed a small area (< 1 m^2) of *L. japonicum* growing in association with multiple areas of *L. microphyllum* (> 50 m^2). This sympatric population occurred at the edge of a bay swamp with a distinct delineation between the

two ferns. *Lygodium japonicum* occurred in a mesic site while *L. microphyllum* occurred in a hydric site on the edge of a bay swamp. The area coverage of both species was relatively small and neither was growing into the canopy, indicating they might have been recent arrivals.

In 2002, a herbarium record (USF #235222) was submitted for *L. japonicum* in St. Lucie County, where *L. microphyllum* is common along roadside canals (Morgan and Overholt, Castanea 70:59–62. 2005). There are herbarium records of *L. japonicum* from five counties in the southern one-third of peninsula Florida including St. Lucie, Martin, Palm Beach, Dade and Collier. Disconnected records for *L. japonicum* between southeast and southwest Florida may be due to low topography and humus soils of the Everglades region or a lack of herbarium submissions. However, it is clear that both *L. microphyllum* and *L. japonicum* are occurring together more frequently in habitats of the central Florida region.

In south Florida, it is unclear why *L. microphyllum* has spread so rapidly but *L. japonicum* still remains a minor floral component in all habitats of south Florida. One hypothesis for the differences in expansion is that *L. microphyllum* thrives in south Florida due to lower topography, humus soils and longer hydrological periods (Volin *et al.*, Divers. Distrib. 10:439–446. 2004), while *L. japonicum* exists in northern Florida due to higher topography, more mesic soils and shorter hydrological periods. Hydric conditions and humus soils may prevent the establishment of *L. japonicum* gametophytes and sporophytes, while drier conditions and sandy soils may prevent the establishment of *L. microphyllum* gametophytes and sporophytes. A second hypothesis may be temperature differences between north and south Florida. A third hypothesis is that *L. japonicum* is less competitive with native plants than *L. microphyllum*. All of these hypotheses are likely interrelated, but it appears that *L. japonicum* has failed to establish in high density in south Florida due to more hydric conditions and humus soils.

As more occurrences of these two invasive ferns occur at the same sites in peninsula Florida, it is possible that hybrids between *L. japonicum* and *L. microphyllum* could be produced. If they do hybridize, there is the potential for heterosis to occur (Hoshizaki and Moran, *Fern Growers Manual*. Timber Press, Portland, OR. 2001; Moran, *A Natural History of Ferns*. Timber Press, Portland, OR. 2004) and further invasion of Florida by the hybrid. While Knoblock *et al.* (Taxon 33:256–270. 1984) reported in a literature review of fern hybrids that there were no *Lygodium* hybrids and speciation in ferns by hybridization appears to be a rare event (Tryon, Bot. Rev. 52:117–156. 1986). Gomez (Brenesia 18:155–170. 1980) reported that hybrids are frequent within mixed populations of *Lygodium* species. For example, two species of *Lygodium* were reported to hybridize forming *Lygodium micans* x *venustum* in Trinidad that occur along different environmental gradients based on pinnae length (Fay, Amer. Fern J. 63:165. 1973; Jermy and Walker, Bulletin of the British Museum, Botany 13:251–276. 1985). In Trinidad, these two species are common with *L. micans* J.W. Sturm most commonly found in forest habitats and *L. venustum* Sw. is found in open sites. Gomez (1980) reports on

an additional hybrid of *Lygodium* spp. (*Lygodium* x *lancetillanum*) as a cross between *L. heterodoxum* Kunze and *L. ventustum*. Clearly, there seems to be potential for hybridization between *L. japonicum* and *L. microphyllum* which could affect future distributions of these species in Florida.

Additional distribution records.—During October 2008, we documented *L. japonicum* growing in the interior of a cypress strand in Naples, Florida (N 26° 12′ 47.7″, W 081° 45′ 52.2″). Infestation of the fern was < 0.05 ha but at least five distinct populations were observed at the site. We observed *L. japonicum* growing at the base of bald cypress [*Taxodium distichum* (L.) Rich.] to heights of 2.5 m, where ground cover was dominated by *L. microphyllum* and the height of *L. microphyllum* was > than 15.0 m. Native plants found in association both species of *Lygodium* at Naples included bald cypress, groundsel tree (*Baccharis halimifolia* L.), wax myrtle [*Myrica cerifera* (L.) Small], myrsine [*Rapanea punctata* (Lam.) Lundell], slash pine (*Pinus elliottii* Engelm. var. *elliottii*), coco plum (*Chrysobalanus icaco* L.), swamp fern (*Blechnum serrulatum* Rich.), royal fern (*Osmunda regalis* L.), and muscadine (*Vitis rotundifolia* Michx.). Two non-native species were also found with the fern and included: creeping oxeye [*Wedelia trilobata* (L.) Pruski] and Brazilian pepper (*Schinus terebinthifolius* Raddi). The interior portion of the site was relatively undisturbed but was surrounded by development on all sides suggesting that hydrological and nutrient alterations have occurred. The site was dry at the time of the survey, but some soil moisture was present. It seems unlikely that *L. japonicum* was planted at this site, but invaded as spores from an unknown source. This species has been in Collier County since the 1930's.

In December 2008, we documented a new, previously unknown location of *L. microphyllum* in Lake County, Florida (N 28° 56′ 40.9″, W 081° 43′ 03.5″). This population of *L. microphyllum* represents an ca. 40 km range increase to the north from the population reported by Pemberton (2003) along the central ridge of Florida, and is ca. 45 km south of a population of *L. microphyllum* documented along the east coast of Volusia County during February 2005 (Wunderlin and Hansen, 2008; Accession # 240261). The infestation was ca. 0.05 ha in size along floodplain habitat at Lake Holly. The fern occurred from the edge of the lake inward to about 25–30 m of the floodplain. The highest density and cover of the fern occurred ca. 5 m from the lakes edge, but scattered populations were found to the north, east and south. The fern was growing up to 4.5 m in height, equaling the height of the tallest native plants, and fertile pinnae were present. Some necrosis (ca. 10% total foliage) was observed due to recent freezes. Native plants associated with the fern at Lake Holly included coastalplain willow (*Salix caroliniana* Michx.), sawgrass (*Cladium jamaicense* Crantz), royal fern, swamp fern, wax myrtle and duck potato (*Sagittaria latifolia* Willd.). The non-native species, Peruvian primrosewillow [*Ludwigia peruviana* (L.) H. Hara], was also present. This new record along the central ridge of Florida may be an indication that *L. microphyllum* will become problematic in the lakes region of north central Florida.

In the extreme southeast part of peninsula Florida, undocumented records of *L. microphyllum* occur ca. 3.5 km west (N 25° 26′ 01.1″, W 080° 22′ 00.7″) of

Turkey Point Nuclear Power Plant in Florida City south of Miami. At this site the fern was well established, growing into Australian pine (*Casuarina equisetifolia* L.) to heights > 10 m and along the edges of mangroves. This population occurs ca. 8 km southeast of Homestead, suggesting that *L. microphyllum* could invade tropical hammocks in Key Largo, which are located ca. 16 km to the south. According to the site manager in 2006 (Geoff West, Florida Power and Lights, pers. comm.), *L. microphyllum* occurs several km south of this site along Card Sound Road. As suggested by Goolsby (Nat. Areas J. 24:351–353. 2004), climates in Caribbean, Central and pats of South America are suitable for *L. microphyllum*, as well as habitats throughout the Florida Keys.

Potential distribution in Florida.—Herbarium records indicate that *L. japonicum* is well established throughout Florida, but is most invasive in the northern part of Florida. After >76 years presence in Florida, it is unknown if *L. japonicum* will become as great a threat to wetland ecotones, forested swamps, and Everglades trees islands in southern Florida that *L. microphyllum* has become. However, the lag time before some non-native plants become invasive can be 70–100 years (Hobbs and Humphries, Conserv. Biol. 9:761–770. 1995). It is in the central Florida region from Ocala (N 29° 07′) to Sebring (N 27° 28′) where *L. japonicum* and *L. microphyllum* are likely to occur together out-competing native vegetation in natural areas. If the two ferns become dominant in the same natural habitat, it is unclear if control efforts (e.g., herbicides, biocontrol) will become more difficult. The Nature Conservancy's Central Florida Lygodium Strategy (CFLS) is designed to work with both private and public landowners in a 12 county region in central Florida region to stop the spread of *L. japonicum* into south Florida and *L. microphyllum* into north Florida (Higgins *et al.*, Nat. Areas J. 27:236–250. 2007; TNC, The Nature Conservancy - stopping the spread in central Florida (http://www.nature.org/wherewework/northamerica/states/florida/science/art23406.html). 2009). The goal of the CFLS is early detection and rapid response to treat *Lygodium* spp. within the 12 county region.

Goolsby (2004) suggested that defoliation of *L. microphyllum* and its inability to grow during periods of cold temperatures may limit its competitiveness with native plants, limiting its range to areas south of Orlando. We hypothesize that *L. microphyllum* will behave like *L. japonicum* during freezes and then re-sprout vigorously in northern Florida during the spring. The growing season is 270 days in northern Florida compared with 330 days along the southeast and southwest coasts of Florida (Henry *et al.*, *The Climate and Weather of Florida.* Pineapple Press, Inc., Sarasota, FL. 1994). It is likely that *L. microphyllum* has now invaded areas between Lake and Volusia Counties to the northeast, though no herbarium records currently exists. This area includes the St. Johns River basin where surface air temperatures are slightly warmer than the surrounding area. How successfully *L. microphyllum* invades and establishes north of Orlando is unknown, but all measures should be attempted to stop its spread into north-central Florida. Stopping the spread of *L. microphyllum* into south Florida rocklands including the Florida Keys is

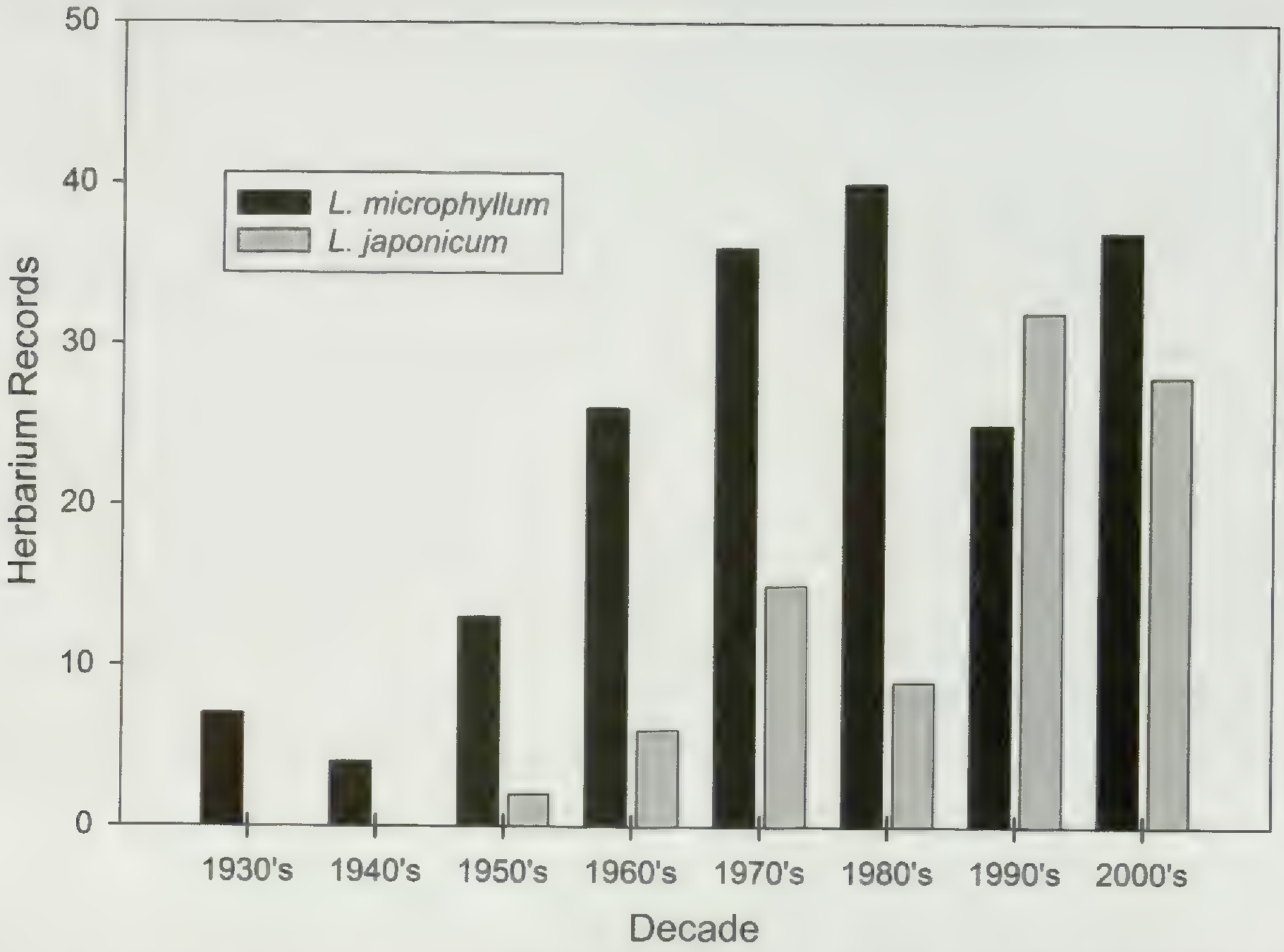

FIG. 1. Number of herbarium records per decade from UF (FLAS), FSU, USF (Wunderlin and Hansen 2008) and Fairchild Tropical Gardens (FTG) for *L. japonicum* and *L. microphyllum* per decade.

equally important because this habitat has numerous endemic plants. The lack of freshwater and humus soils may limit the spread of *L. microphyllum* in the Florida Keys.

It is clear that *L. microphyllum* has spread into USDA Cold Hardiness Zone 9A in Florida where the average minimum temperature can range between −6 to −3 °C. North Florida receives 4–9 days in December and 5–11 days in January in which the temperatures drops below 0 C, while south Florida only receives 0–1 days in both December and January in which the temperatures drops below 0 °C (Henry *et al.*, 1994). Potentially, *L. microphyllum* could spread along the southeast coast of Florida northward to the Jacksonville/Georgia border, north along the central ridge to Ocala, and along the west coast of Florida to the northern edge of Citrus County or southern Levy County. This is the potential northern range of *L. microphyllum* suggested by Pemberton (2003) and Goolsby (2004). In lab experiments, we observed young sporophytes of *L. microphyllum* resprout after three hours at −2.2 C and spore germination following 12 hours at −2.2 °C (Hutchinson and Langeland, unpubl. data).

Conclusions.—Based on herbarium records (FLAS, FSU, USF and FTG), there were increased reports of *L. japonicum* during the 1960's and *L. microphyllum* in the 1990's (Fig. 1). Considering that *L. japonicum* was first documented in Florida ca. 1930, it appears that within 30 years of being

documented it had began to spread across the state, possibly from multiple introductions and increased propagule pressure. Yet, *L. japonicum* has failed at this point in time to establish and invade natural areas of south Florida. We observed a similar invasive pattern based on herbarium records of *L. microphyllum*, in which increased herbarium records were documented ca. 30 years after it was first reported in Florida. However, it is unknown if *L. microphyllum* was introduced into more than two sites in Florida. It was reported that *L. microphyllum* was sold at Royal Palm Nursery in Manatee County from 1888–1930, but evidence from a photo in the nurseries catalogue and a description of its hardiness indicates it was *L. japonicum* (Pemberton and Ferriter, 1998).

Based on our observations and those of Lockhart (2007), coverage of *L. microphyllum* dominates *L. japonicum* in sites where these species are sympatric. Based on limited observations in Florida, these two species can occur in close proximity, but are separated by distinct ecotones based on slight changes (< 1.0 m) in elevation. There is evidence that introduced species and habitat disturbance within a new range may create ideal situations for hybridization and possibly heterosis in ferns (Walker, Evolution 12:82–92. 1958; Fay, 1973; Gomez, 1980; Kentner and Mesler, Am. J. Bot. 87:1168–1174. 2000). Habitat disturbances are numerous in Florida and two invasive climbing ferns are now present in close proximity. Both *L. japonicum* and *L. microphyllum* foliage exhibit high necrosis (i.e., up to 100%) from hard frosts, but re-sprouting occurs from rhizomes below the soil, indicating these species can tolerate temperatures ≤ 0°C. *Lygodium microphyllum* may exhibit the same tolerances as *L. japonicum* with regard to temperature, while invading further into north Florida. It remains unclear if *L. japonicum* will invade natural areas of southern Florida, but with multiple introductions throughout the state and the potential for long distance dispersal by spores, it is possible that this species may increase at an exponential rate.—Jeffrey T. Hutchinson and Kenneth A. Langeland, University of Florida, Agronomy Department, Center for Aquatic and Invasive Plants, 7922 NW 71st Street, Gainesville, Florida, U.S.A.

Carbon Isotope ($\delta^{13}C$) Signatures of Rheophytic *Dipteris lobbiana*.—As with most plant groups, ferns show their highest diversity in the tropics (Mutke and Barthlott, Biol. Skr. 55:521–537. 2005). *Dipteris lobbiana* (Hook. *f.*) Moore is a fern species found commonly growing in riparian habitats throughout the tropical island of Borneo where it is often strongly attached to rocks along and within streams (Fig. 1). It belongs to the family Dipteridaceae which has two genera and nine species found only in the Palaeotropics (Kramer, *in* K. U. Kramer & P. S. Green eds. *The Families and Genera of Vascular Plants Vol. 1. Pteridophytes and Gymnosperms*: 99–101. Springer-Verlag, Berlin, Germany. 1990).

FIG. 1. *Dipteris lobbiana* growing adjacent to streams at (a) Bako National Park, Sarawak, Malaysia and (b) Barito Ulu, Central Kalimantan, Indonesia.

The maintenance of a positive carbon balance is important for the growth and survival of all plant species and is related to the amount of carbon fixed per unit of water lost (water use efficiency; WUE). As *D. lobbiana* grows as a facultative rheophyte and has access to large sources of water, I hypothesised that it would have a low WUE. A simple way to test this hypothesis is through analysis of its stable carbon isotope values.

Stable carbon isotope values ($\delta^{13}C$) of plant tissues are controlled by the primary carboxylating enzyme used in photosynthesis, the isotopic composition of the atmospheric CO_2 taken up by the plant, and the ratio of the internal to external concentration of CO_2 which is primarily controlled by stomatal opening (O'Leary, BioScience 38:328–336. 1988; Farquhar *et al.*, Annu. Rev. Plant Physiol. Plant Mol. Biol. 40:503–537. 1989). Measures of leaf $\delta^{13}C$, which integrate responses over the lifetime of the leaf, can be used as a measure of WUE. If we assume that the isotopic composition of the atmospheric CO_2 is the same at all sites studied, the foliar isotopic value is therefore dependent upon the relative importance of the leaf stomatal conductance (*G*s) and maximum rate of photosynthesis (A_{max}). When water levels are low, *G*s is reduced and there will be slower uptake of the heavier ^{13}C isotope; this will lead to leaves having less negative $\delta^{13}C$ values. Equally, where A_{max} is higher, ^{13}C will be taken up more readily and foliar $\delta^{13}C$ will be more negative. In sum, factors reducing the supply of CO_2 through, for example, stomatal closure lead to less negative $\delta^{13}C$ values.

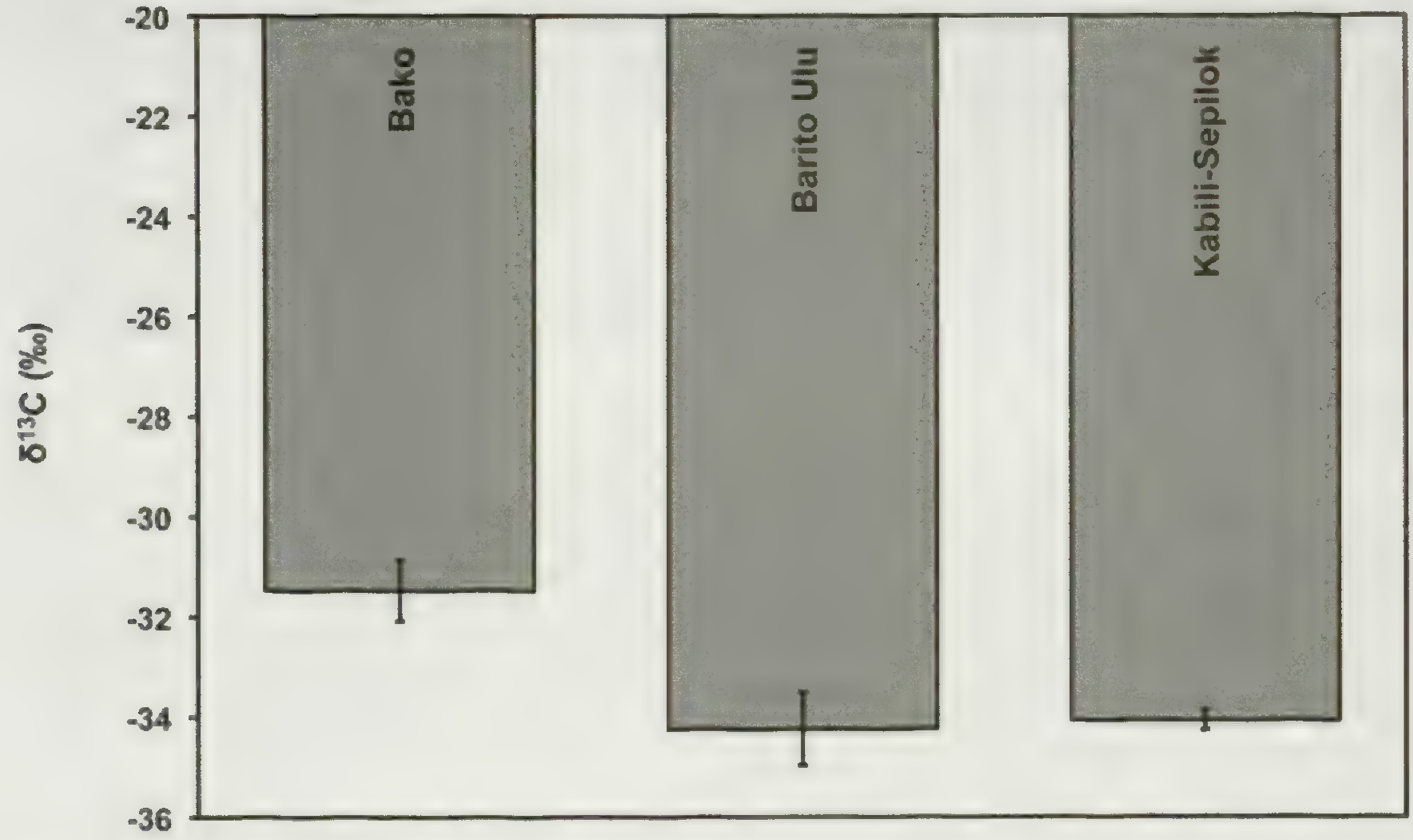

FIG. 2. Foliar carbon isotope ($\delta^{13}C$) values of *Dipteris lobbiana* from three sites in Borneo.

Flanagan *et al.* (Oecologia 111:481–489. 1997) showed that where water levels were not limiting there were more negative foliar $\delta^{13}C$ values in plants growing in mesic 'hanging garden' communities in Utah (USA) where water continually seeps through the porous rocks and is easily available for these plants. In contrast, plants from desert communities can have much less negative $\delta^{13}C$ values indicating the severe water stresses they face (Winter *et al.*, Oecologia 25:115–123. 1976; Ehleringer *et al.*, Rev. Chil. Hist. Nat. 71:79–86. 1998).

I examined foliar carbon isotope values to determine the WUE of *D. lobbiana* from three sites in Borneo. I hypothesised that as this fern is rheophytic it would have very negative $\delta^{13}C$ values and that there would be no difference in $\delta^{13}C$ values between the three sites.

Leaf samples were taken in 2006 from Bako National Park in Sarawak (1° 43′ N; 110° 28′ E), Barito Ulu in Central Kalimantan (0° 06′ S; 114° 0′ E) and Kabili-Sepilok Forest Reserve in Sabah (5° 51′ N; 117° 58′ E) from small- to medium-sized individuals ($n = 3$ per site). Voucher specimens from Bako and Kabili-Sepilok are in TCD with Kabili-Sepilok duplicates in SAN. Samples were dried, ground, and analyzed on a Finnegan Delta^{plus} IRMS interfaced with a Carlo Erba elemental analyser via a Conflo III.

The carbon isotope values were negative (mean value across all sites of −33.3‰; Figure 2) as expected for plants growing in forest environments due to the low light levels found around the shaded understorey streams (Farquhar *et al.*, Annu. Rev. Plant Physiol. Plant Mol. Biol. 40: 503–537. 1989) and also the incorporation of respired carbon dioxide which is depleted in ^{13}C relative to well mixed atmospheric conditions (Buchmann *et al.*, Oecologia 110:120–131.1997). This mean value of −33.3‰ is comparable to the mean value of

−33.8‰ for terrestrial fern species found at La Selva in Costa Rica by Watkins Jr. *et al.* (Oecologia 153:225–232. 2007). The value is not unusually negative and suggests that there are no major differences in WUE between this rheophytic fern species and many terrestrial fern species although analyses of other species from the Borneo study sites would allow confirmation of this suggestion. It also confirms the generally more conservative water use strategies of ferns when compared with angiosperms (Brodribb and Holbrook, New Phytol. 162:663–670. 2004).

Foliar samples from Bako had a significantly less negative foliar $\delta^{13}C$ values than samples from the other two sites ($F_{2,6} = 7.65$, $p = 0.022$; Fig. 2). This was somewhat surprising given that this site has the highest annual rainfall (Bako: 4025 mm, Barito Ulu: 3800 mm, Kabili-Sepilok: 3000 mm) but could be due to the desiccating winds of the more coastal environment. There is also a possibility that stream flow may be less regular at this site and is having an effect on the water relations of this fern species.

This study has shown that *Dipteris lobbiana* does not have a particularly low WUE and that, even for this potentially non-water stressed fern species, there are differences in $\delta^{13}C$ between sites suggesting that water limitation of rheophytic ferns may occur if stream flow is reduced.

I thank Robbie Goodhue (Trinity College, Dublin) for his help with isotope analysis, the Indonesian and Malaysian authorities for permission to work in their respective countries and Muhammed Mansur (Herbarium Bogoriense) for collecting samples from Barito Ulu.—FRANCIS Q. BREARLEY, Department of Environmental and Geographical Science, Manchester Metropolitan University, Chester Street, Manchester, M1 5GD, UK, f.q.brearley@mmu.ac.uk.

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AMERICAN FERN JOURNAL

Volume 100

Number 2

April–June 2010

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American Fern Journal 100(2):71–79 (2010)

Comparative Morphology of Ligules of Three Indian Species of *Selaginella*

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Abstract.—The ligule morphology in three Indian species of the genus *Selaginella*, e.g., *S. bryopteris*, *S. repanda* and *S. panchghaniana* is described for the first time. Though the leaves (ventral and dorsal) in all the three species are typically normal, some dorsal leaves in *S. panchghaniana* have two ligules, bifid tips and lobed bases. The form of ligules is variable within each species but the overall shape (outline) is constant. The size of ligules may be influenced by environmental conditions. The presence of glandular cells at the margin of the tip of ligules indicates a water secreting function.

Key Words.—*Selaginella*, leaves, ligule, lobed, shape and glandular cells

The ligule is a membranous tongue-like structure present on the adaxial surface of the leaf at the junction of leaf and stem. It is the characteristic feature of a number of extinct arborescent lycopsids, the Devonian herbaceous *Leclercqia* and extant genera such as *Sellaginella* and *Isoetes*. Bower (1959) realizing the importance of this small structure divided Lycopsida into the two groups Eligulatae and Ligulatae. Hofmeister (1851) was the first botanist to report the presence of ligules in the genus *Selaginella*. Since these reports, a number of workers (McNab, 1887; Campbell, 1895; Velenovsky, 1905; Verdoorn, 1938; Von Goebel, 1930) have given reference to or brief mention of ligules in their description of the genus.

Harvey Gibson (1896) first gave a detailed account of the structure, development and function of ligules of *Selaginella*. Ball (1925) reported the presence of double ligules within a single sporophyll in *S. martensii* Spring. Vink (1953) described the lobed ligules in *S. caulescens* (Wall.) Spring. Hsu (1937) and Lafont and Lemoigne (1965) gave the brief descriptions of the ligules of *S. sinensis* (Desv.) Spring and *S. willdenovii* (Desv.) Baker, respectively. Dahlen (1988) made a detailed observation of the ligules both on stems and branches of 11 species of *Selaginella* from Hong Kong and concluded that ligules of mature lateral leaves on branches are the least variable and most representative of a specimen. He also concluded that despite some variability, ligule shape may be independent of other morphological features and/or environmental influences and that it may be used to distinguish species.

Webster (1970) successfully developed aberrant leaves on the angle-shoot meristem in *S. martensii*. Kohlenback and Geier (1970) studied the function of

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ligules of *S. kraussiana* A. Braun and reported that the ligules were found to absorb Berberine sulfate in the apical region where the cuticle is thin. Bilderback (1987) found the association of mucilage in the ligules of a number of species of *Selaginella*.

Horner and Beltz (1975) described the development and fine structures of ligules in *S. pilifera* A. Braun and *S. uncinata* (Desv.) Spring. Lerbs (1974), Sigee (1974, 1975, 1976) and Bilderback and Slone (1987) described the ultrastructural and physiological details of ligules in *S. martensii* and *S. kraussiana* respectively. Jagels and Garner (1979) observed the variation in callose deposition in the ligules of *Selaginella*.

Shaw and Hickey (2005) have given a comparative account of *Selaginella* and *Isoetes*. The basic organization of the ligules of *Selaginella* and *Isoetes* is same, however the ligule of *Selaginella* is much simpler than that of *Isoetes* and lack elaborate glassopodium.

The survey of literature on *Selaginella* has revealed that very little attention has been paid to the studies of the ligules. As a result, this structure remains neglected especially by the Indian workers (Dixit, 1992) and others. No significant advancement has been made in the knowledge of *Selaginella* ligules since 1987, with the exception of a review by Pant *et al.* (2000) and a brief mention by Taylor *et al.* (2009). The present study was designed to increase our knowledge of ligule structure in *Selaginella*.

Material and Method

The specimens of different species of *Selaginella* Beauv. were collected from a number of localities in India: *S. bryopteris* (L.) Baker from Bhilaigarh (CH), Jargodam, Ahrora and Sidhnath fall (U.P.); *S. repanda* (Desv. ex Poir), from Matkuli and Apsravihar (M.P.); and *S. panchghaniana* Dixit, from Khandala, Panchgani Table-land and Bhose (MH). Habitat and the morphometric details were observed in the field. Fresh materials were fixed in FAA for further examination in the laboratory. Ten herbarium vouchers were prepared from each locality of each species and the specimens were deposited in Duthie Herberium, Department of Botany, University of Allahabad, Allahabad. The terminology followed in the paper is adopted from Shaw and Hickey (2005).

For the present investigation, the ligules of five plants per population and five leaves from each specimen were randomly selected for the studies of apical, middle and basal regions of both ventral and dorsal leaves. The ligules were dissected out from basal region of the leaf and mounted in Glycerin jelly. The size (length and breadth) of ligules were measured under light microscope and photographed with a Leica DMLB DC300 microphotographic microscope.

Results

Selaginella bryopteris is a non-rhizophoric species, the other two, *S. repanda* and *S. panchghaniana*, are rhizophoric species.

Ligules are present on both ventral and dorsal leaves of all of the three species of *Selaginella* (Fig. 1). In all three species both ventral and dorsal leaves are of the normal type, i.e., single leaf with single ligule, single tip and single base. However, sometimes in *S. panchghaniana* the dorsal leaves possess two ligules, bifid tips and two bases (i.e., single leaf with two ligules) (Fig. 1F, G). Both ligules are nearly the same size (Fig. 1F), but occasionally one of them is larger and than other (Fig. 1G).

In *S. bryopteris* the ligules are situated in a shallow cavity at the base of leaves with less marked swollen regions, but in *S. repanda* and *S. panchghaniana* the ligules appears just above the surface of leaves at their base. The position of ligules is parallel to mid-vein of leaves in *S. bryopteris* and *S. repanda* but oblique in *S. panchghaniana*.

The shape of ligules is variable. It is cup-shaped or somewhat fan-shaped in *S. bryopteris* (Fig. 2A–D), but horn-shaped or tongue-like in *S. repanda* (Fig. 2E–I) and only tongue-like in *S. panchghaniana* (Fig. 2J–L; Fig. 1A–C). Though the shape (outline) of the ligule is constant within species, minor morphological variation has been recorded among the ligules of the same specimen. Each ligule is composed of two major parts, glassopodium and tongue. The tongue consists of two sections, namely the bulbous base and the tip or neck. The glassopodium is represented by a few cells present at the base of the ligule. The cells of the bulbous base are hexagonal and dark in color in comparison to that of the tip or neck. The cells of tip or neck section are more elongated. The tip of ligule of *S. bryopteris* is multi- to tri- lobed and the margin of the tip is crenate (Fig. 2A–D), and those of *S. repanda* are tri-, bi-, or unlobed and the margin of the tip is obtuse (Fig. 2E–I). The ligules of *S. panchghaniana* are unlobed to vary rarely bilobed. The margin of the tips of ligules varies from obtuse to acute (Fig. 2 J–L; Fig. 1 A–C).

The size (length and breadth) of ligules varies among the species. It is 285–105 μm (L) × 457.5–82.5 μm (B) in *S. bryopteris*; 285–142.5 μm (L) × 135–67.5 μm (B) in *S. repanda* and 345–105 μm (L) × 105–37.5 μm (B) in *S. panchghaniana* (see Table 1).

Discussion

Harvey Gibson (1896) provided a detailed description of the structure, development and function of ligules in 50 species of *Selaginella*. He had remarked that "considerable variation occurs in the precise form of the ligule, even in the same plant; but, on the whole, within certain limits, the outline is maintained fairly constantly for the species". Our studies of *Selaginella* support the observations of Harvey Gibson (1896) and Dahlen (1988). The ligule shape of different species of *Selaginella* is characteristic and likely to be helpful in taxonomic delineation.

Normally each ventral and dorsal leaf is provided with one tip, one base and one ligule (Fig. 1D, E). However, we have observed that in some dorsal leaves of *S. panchghaniana* bifid tips, two inseparable basal lobes and two ligules of equal or unequal size (Fig. 1 F, G), are present. Previously, Ball (1925) noticed

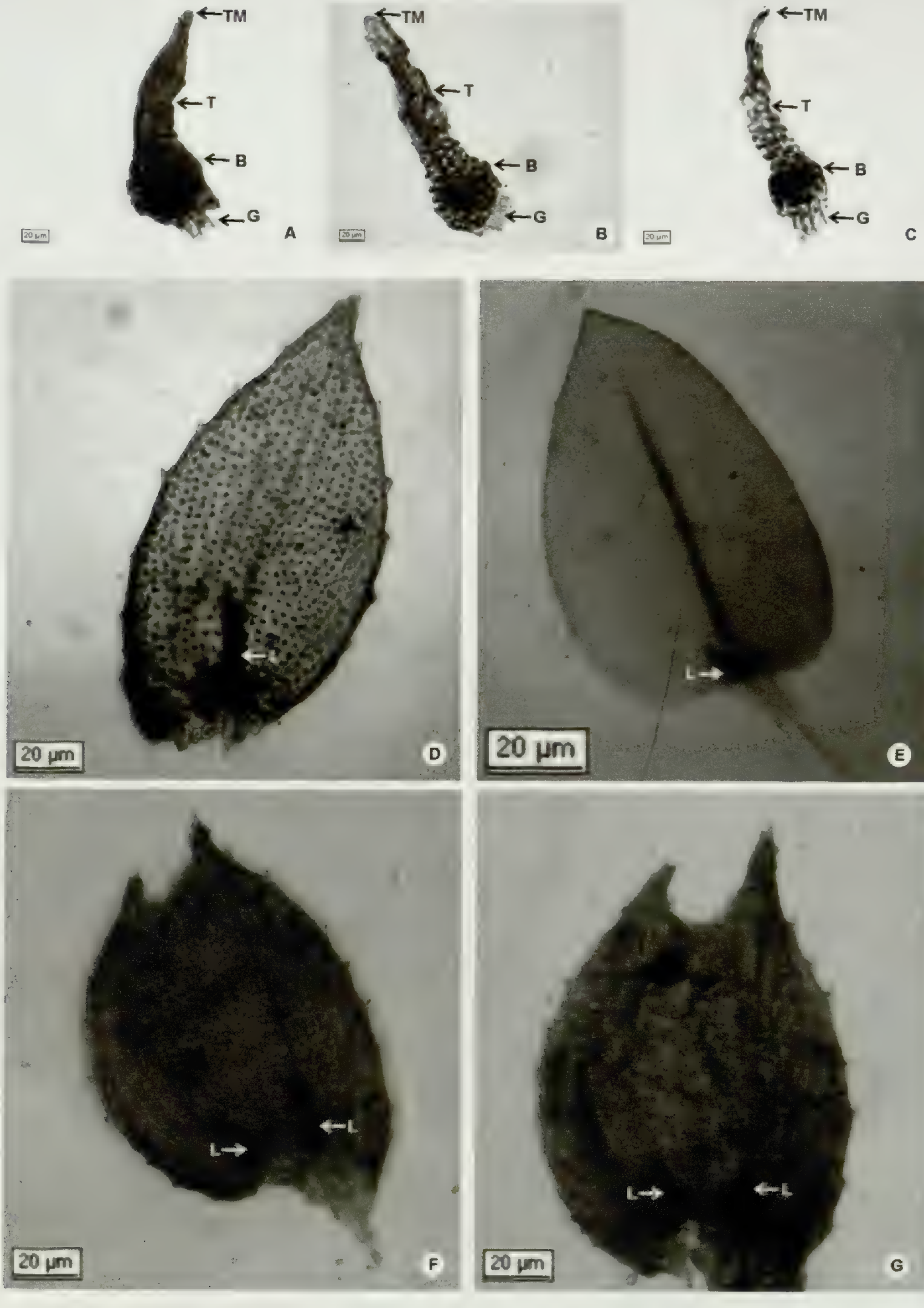
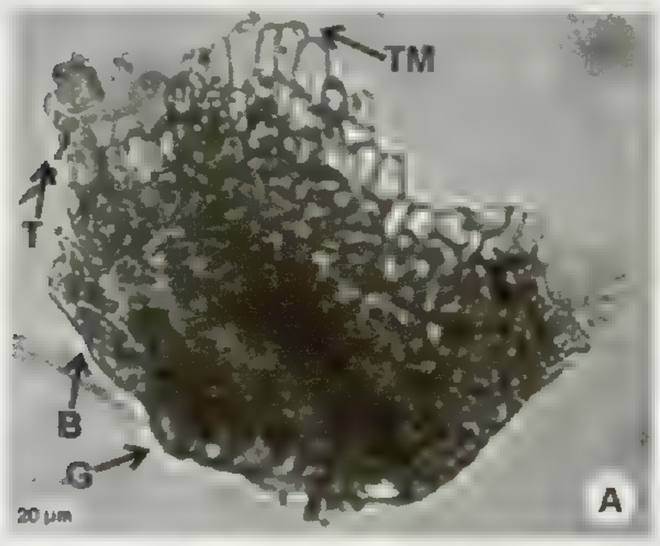
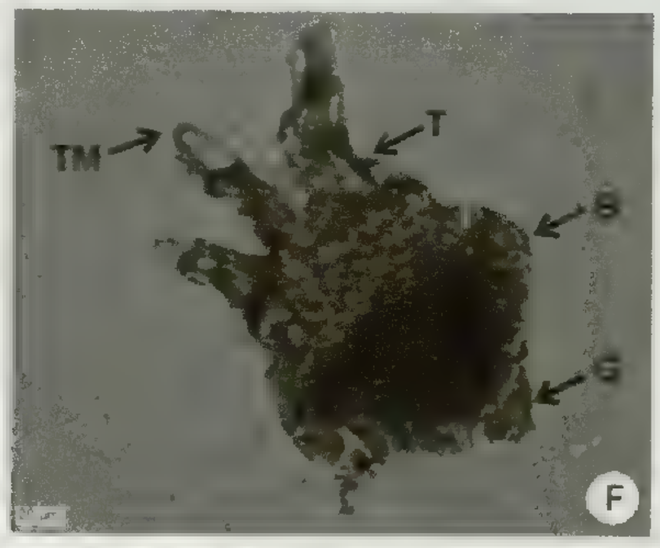
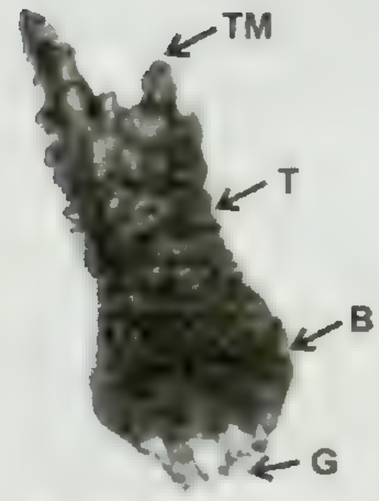
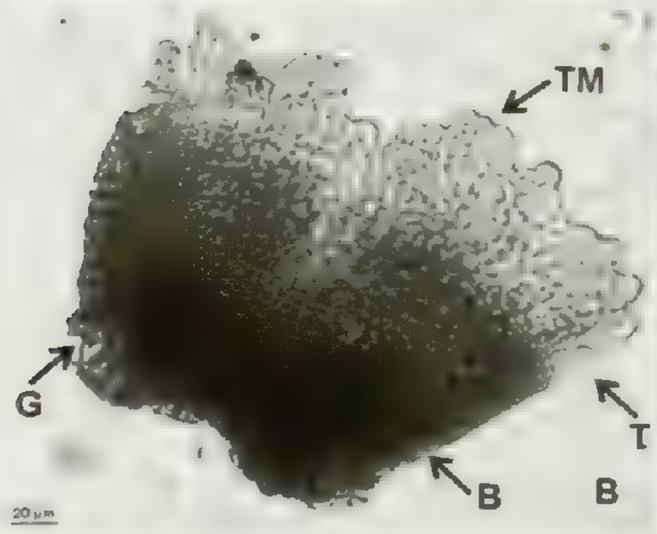


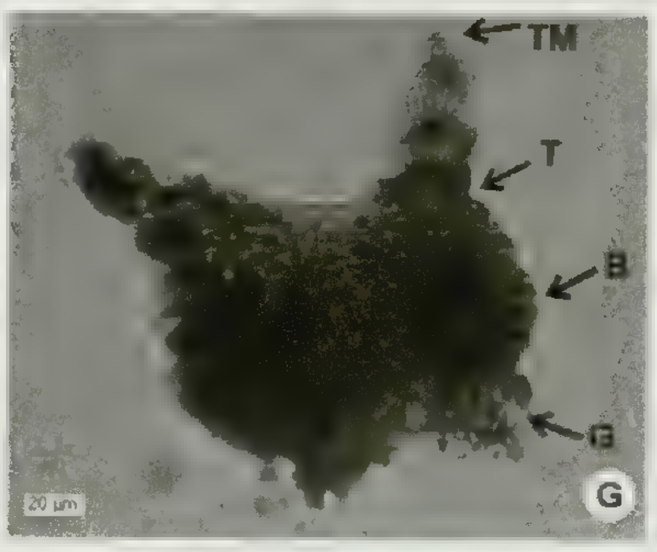
FIG. 1. A–C. Ligules of *S. panchghaniana*. D–G. Leaves of *S. panchghaniana*. A–B. Tongue-like with unlobed and obtuse margin. C. Tongue-like with unlobed and acute margin. D & E. Dorsal and ventral leaves with single ligule, respectively. F & G. Dorsal leaves with double ligule of equal and unequal size, respectively. TM = Tip margin, T = Tip part, B = Basal part, G = Glassopodium, L = Ligule.

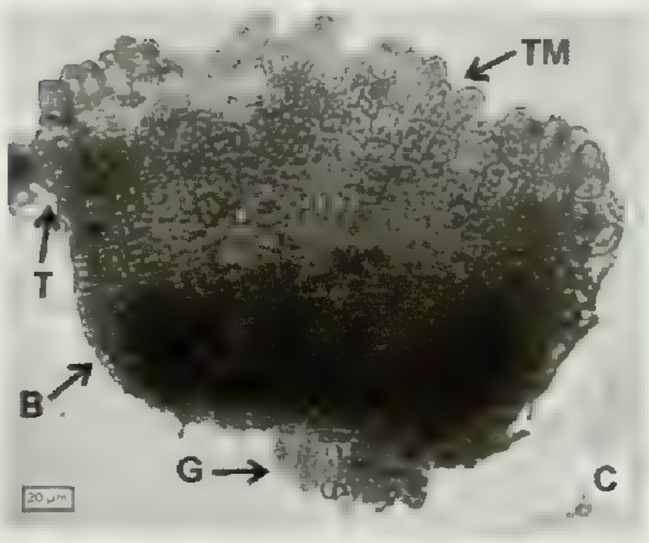


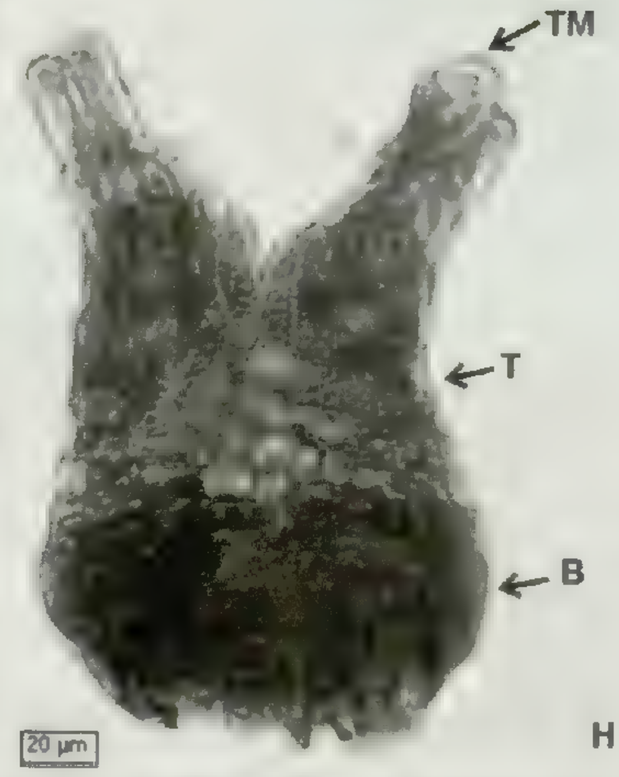


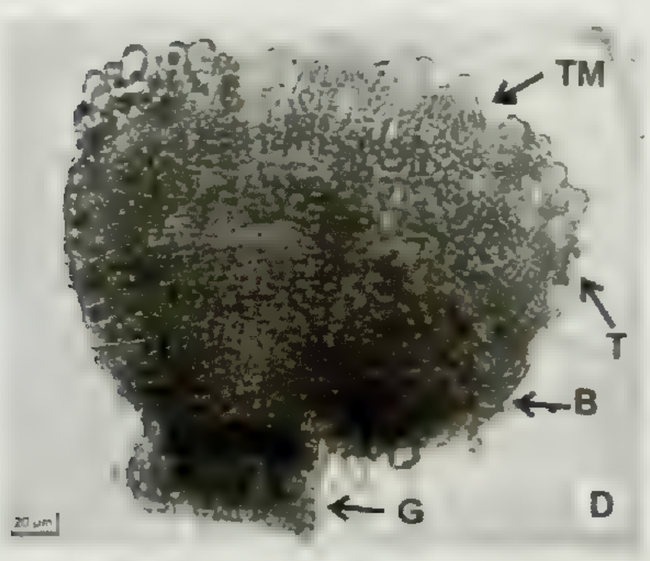












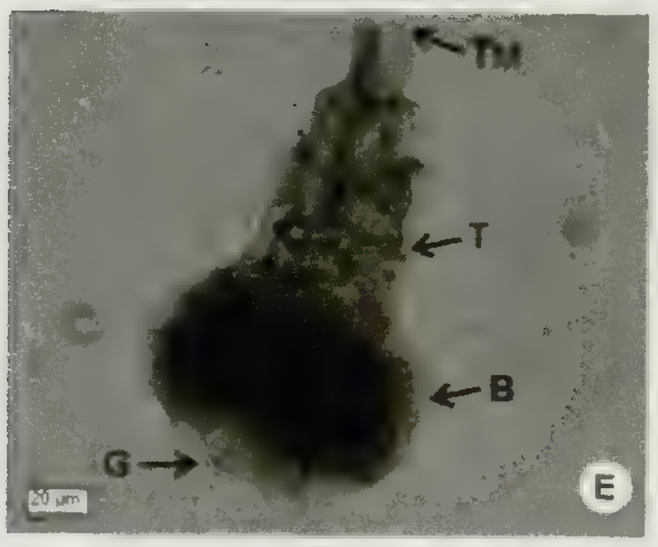


FIG. 2. Ligules. A–D. *S. bryopteris*; E–I. *S. repanda* and J–L. *S. panchghaniana*. A. Cup-shaped with trilobed tip and crenate margin. B–D. Cup-shaped with multilobed tip and crenate margin. E. Tongue-like with unlobed an obtuse margin. F. Heart-shaped with trilobed tip and obtuse margin. G & H. Heart-shaped with bilobed tip and obtuse margin. I. Tongue-like with unlobed and obtuse margin. J. Tongue-like with bilobed tip and acute margin. K–L. Tongue-like with unlobed and obtuse margin. TM = Tip margin, T = Tip part, B = Basal part, G = Glassopodium.

TABLE 1. Showing the differences in size (length and breadth) of ligules of three species of *Selaginella*.

Name of species	Habitat condition	Shape, tip and margin of tip of ligules		Ligules of ventral leaves. Min. (μm)	Max. (μm)	Av. (μm)	S.D.	CV.	Ligules of dorsal leaves. Min. (μm)	Max. (μm)	Av. (μm)	S.D.	CV.
S. bryopteris	Highly dry and open	Cup–shaped or fan-shaped; multi to tri- lobed; crenate.	L.	165	285	216.5	23.2	10.7	165	255	210	22.9	10.9
			B.	150	420	260.9	63.4	24.3	120	457.5	245.1	55.2	22.5
	Dry and open	As above	L.	135	225	189.1	20.3	10.7	105	240	177.5	21.6	12.2
			B.	127.5	225	224.1	55.8	24.9	82.5	300	197.3	45.5	23.1
S. repanda	Semi wet and open	Horn- shaped and tongue-like; tri, bi and unlobed; obtuse.	L.	142.5	285	207.1	33.8	16.3	142.5	270	212.5	28.2	13.3
			B.	67.5	135	103.9	18.3	17.6	67.5	120	93.5	13.2	14.2
S. panchghaniana	Wet and shaded	Only tongue-like; unlobed, rarely bilobed; obtuse to acute.	L.	105	345	214.6	47.2	21.9	105	332.5	220.5	41.5	18.8
			B.	45	97.5	69.2	12.7	18.4	37.5	105	60.1	12.3	20.4

L. = Length, B. = Breadth.

two cases of the presence the two ligules of unequal size in a single sporophyll of *S. martensii* in classroom slides. Webster (1970) successfully developed aberrant leaves from an excised angle shoot meristem with 2–5 tips, as well as mid-ribs and single or double ligules of equal size. The presence of dorsal leaves with more than one tip and ligule in Indian species of *Selaginella* is in agreement with earlier reports. However, these reports are confined either to classroom examination of slides or cultural conditions. We report, for the first time, the occurrence of double ligules in *S. panchghaniana* growing *in situ*. This indicates that some of the species of *Selaginella* have the potential to produce more than one ligule in a single microphyll or sporophyll.

Selaginella bryopteris is characterized by the presence of a radial shoot without aerial roots (rhizophores) and the presence of ligules in a shallow cavity at the leaf base indicates its retention of ancestral traits. Bower (1959), on the basis of radial shoots and absence of aerial roots had regarded that "*S. spinulosa* may be a primitive species of *Selaginella*". This is further supported by Korall *et al.* (1999). They studied generic / sub-generic relationships of Selaginellaceae based *rbcL* gene sequences, and found that the group of *Tetragonostachys* has more extended distribution of rhizophores between branches in many places along the stem and concluded that "the relationships of *Tetragonostachys* indicate that the extended distribution of rhizophores is a derived characteristic". Maslen (1898) and Grierson and Bonamo (1979) also reported that the ligules of *Lepidodendron* and *Leclerquia* respectively, arise from the shallow depression on the adaxial surface of leaves. In our observation the presence of a radial shoot without aerial roots, and ligules in shallow cavity of *S. bryopteris* point out its ancestral nature.

In *S. repanda*, both ventral and dorsal leaves are of normal type with horn-shaped or tongue-like ligules. Fan-shaped and tongue-like ligules have been described by many workers (Harvey Gibson, 1896; Vink, 1953; Horner and Beltz, 1975; and Dahlen, 1988), but no one has described horn-shaped ligules. Ours is the first report of horn-shaped ligules in *S. repanda*.

The plants of *S. bryopteris*, *S. repanda* and *S. panchghaniana* are found growing in different ecological conditions. The ligules of xerophytic species (*S. bryopteris*) are tougher, thin and light colored, generally with more than one lobe of which the tip margins are crenate with many glandular cells (Fig. 2 A–D). The ligules of *S. repanda* are loose, thick, dark colored and tri-, bi-, or unlobed, and tip margins are obtuse with few glandular cells (Fig. 2 E–I). The ligules in the mesophytic species (*S. panchghaniana*) are loose, thick, dark colored, and unlobed. The ligules are occasionally divided and their tips are obtuse to acute, with fewer glandular cells than *S. repanda* (Fig. 2 J–L; Fig. 1 A–C). Our observations on ligules of xerophytic species is similar to those of *S. cauliscens* and *S. rupestris* described by Vink (1953) and *S. lepidophylla* described by Jagels and Garner (1979). These species are xerophytic and the structure of the ligules is almost same in all the species. The ligules of xerophytic species are more prominent and robust than other species. It suggests a structural adaptation due to the stressful conditions and greater water demand (see Table 1). The presence of glandular cells at the margin of

the tips of ligules further supports that the major function of ligules may be secretion.

No specific function of the ligule is known but a number of hypotheses have been proposed to elucidate the function of ligules in *Selaginella*. The hypothesized function of ligules can be grouped into three categories: organ of absorption (Mc Nab, 1887; Kohlenbach and Geier, 1970); secretion of water or mucilage (Hofmeister, 1851; Harvey Gibson, 1896; Maslen, 1898; Foster and Gifford, 1959; Bierhorst, 1971; Bilderback and Slone, 1987; and Bilderback, 1987); production of callose (Jagels and Garner, 1979) or complex carbohydrate (Sigee, 1976). However, Lerbs (1974), Lafont and Lemoigne (1965), Sigee (1974, 1975, 1976) and Horner & Beltz (1975) could not detect any actual exudations of secreted substances in ligule. Sigee (1974) said that ligule is a vestigial organ, having lost its secretory function during the course of evolution. Our observations support the view that the ligule is physiologically active and may be involved in the secretion of water to keep the young leaf moist.

The present study reveals that the morphology of ligules provide an additional taxonomic tool for the delimitation of *Selaginella* species. The varied environmental conditions are associated with the morphometric features of ligules within the plants of same species.

Acknowledgments

The authors are grateful to Prof. N. Bhowmik, Head, Department of Botanty, University of Allahabad, for providing research facilities. We are thankful to Dr. S. Chandra, Lucknow, Prof. D. K. Chauhan and Dr. S. P. Tiwari, Allahabad, for valuable suggestions and Prof. G. L. Tiwari, Allahabad, for helping in microphotographs. Thank are also due to Dr. (Mrs) P. Nisha and Dr. Santosh Namphy, Department of Botany, St. Joseph College, Calicut, for their help in collection and identification of plant material. We also thank the reviewers for valuable comments and suggestions. The first author is thankful to UGC for providing financial support through University of Allahabad, Allahabad.

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Comparative Anatomy of the Nectaries of *Aglaomorpha* and *Drynaria* (Polypodiaceae)

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ABSTRACT.—The anatomy of the foliar nectaries of four drynarioid species (*Drynaria quercifolia*, *D. rigidula*, *Aglaomorpha coronans* and *A. acuminata*) is described. The nectaries of all four species are vascularized modifications of the leaf blade and are distinct from surrounding tissue. They are composed of densely staining tissue with small isodiametric cells and reduced intercellular spaces. The nectaries of *D. quercifolia* and *A. coronans* occur as patches of tissue on the leaf blade. In contrast, the nectaries of *D. rigidula* and *A. acuminata* are stand-alone structures. The nectaries of *A. acuminata* represent the most specialized nectaries yet described among the ferns. The contribution of these data to cladistic analyses of *Drynaria* and *Aglaomorpha* is discussed.

KEY WORDS.—Nectary anatomy, *Aglaomorpha*, *Drynaria*

Drynarioid ferns are a monophyletic grouping of 30 species now classified in two genera, *Aglaomorpha* and *Drynaria* (Roos, 1985; Janssen and Schneider, 2005) in the Polypodiaceae. The ferns are epiphytic, they occur in the paleotropics (Copeland, 1947; Holttum, 1968) and many species have humus-collecting nest leaves in addition to photosynthetic foliage leaves (Holttum, 1968; Janssen and Schneider, 2005). A distinctive characteristic of drynarioid ferns is the presence of specialized nectar-secreting glands on the leaves (Copeland, 1947; Holttum, 1968; Koptur *et al.*, 1982; Elias, 1983; Roos, 1985; Janssen and Schneider, 2005).

Drynarioid nectaries are described as translucent spots located close to the junction of the costa/rachis and occurring in the lobes of petiolar wings. The *Aglaomorpha acuminata* (Willd.) Hovenkamp nectary is situated on a "specialized quadrangular extension" (Roos, 1985). Drynarioid nectary anatomy and morphology is rarely noted in detail. It has been described only briefly as a grouping of columnar cells with an associated plexus of vascular tissue (Nayar, 1961).

> "Nectaries occur irregularly distributed close to the midrib and some of the main lateral veins of the lamina. The nectaries are irregularly oval or circular, pellucid to yellowish areas in the lamina, and in very young leaves secrete a viscous, sweet, sometimes bad smelling liquid. They are composed of compactly placed glandular cells which are columnar and noncholorophylous. A vascular plexus formed by intersecting veinlets and composed of diffused vascular tissue occurs towards the center of each nectary."

Additionally, the distribution of nectaries on leaves of ten drynarioid species has been described and three distribution patterns were identified (Zamora and Vargas, 1974). The secretion of liquid from various drynarioid

species, including *Drynaria rigidula* (Sw.) Bedd. has been confirmed to be nectar (Koptur *et al.*, 1982).

In this study, the anatomy of the foliar nectaries of four species of drynarioid ferns (*A. acuminata*, *A. coronans* (Wall. ex Mett) Copel., *Drynaria quercifolia* (L.) J. Sm. and *D. rigidula*) is described. These data are compared within the group and to nectaries of *Pteridium*, another fern species with nectaries. The data are then discussed in the context of the most recent cladistic analysis (Janssen and Schneider, 2005).

Nectaries vary widely in anatomy, secretion method and location on the plant (Fahn, 1979; Elias, 1983). Various types of extrafloral nectaries have been described based on anatomical structure, including a number of which occur on foliage leaves, like those of drynarioid ferns (Zimmerman, 1932). This diversity of nectaries is strong evidence that they have evolved many times throughout the history of flowering plants and ferns (Elias, 1983).

Other ferns that bear nectaries include the common bracken fern (*Pteridium*) (Darwin, 1877; Page, 1982; Power and Skog, 1987; Rumpf *et al.*, 1994) and *Cyathea* (pers. comm. White, 2007). In *Polypodium*, *P. myriolepis* Christ, *P. pyrrholepis* (Fée) Maxon, *P. rosei* Maxon, *P. sanctae-rosae* (Maxon) C.Chr. and *P. thyssanolepis* A.Br. ex Klotzsch were also observed to secrete nectar (Koptur *et al.*, 1982). Among the ferns, the anatomy of nectaries of *Pteridium* has been studied in the most detail.

Based on combined molecular and morphological evidence, a recent study concludes that the genus *Drynaria* is paraphyletic and that *Aglaomorpha* is a monophyletic group derived from within it (Fig. 1) (Janssen and Schneider, 2005). Despite these findings, the morphological data, alone, do not support a paraphyletic *Drynaria*. Although the presence and position of drynarioid fern nectaries are used as character states in the analysis (Janssen and Schneider, 2005), it does not take into account possible differences in nectary anatomy. As anatomical data are important for phylogenetic reconstruction (Kaplan, 1984), using nectary anatomy as a character state may help to clarify the morphological data set.

Materials and Methods

Young nectary-bearing leaves of each of three species (*Drynaria quercifolia*, *Drynaria rigidula*, *Aglaomorpha acuminata*) were collected from the Duke University greenhouse. Due to the absence of young material, mature leaves were used for the fourth species, *Aglaomorpha coronans*. The nectaries were cut from the leaves of each species and then preserved in formalin-acetic acid alcohol (FAA) and dehydrated in a tertiary butyl alcohol series (Johansen, 1940). Voucher specimens for the species are deposited in the Duke Herbarium (DUKE).

The leaf material of *D. quercifolia*, *D. rigidula*, and *A. speciosa* was imbedded in paraffin using standard techniques (Johansen, 1940). Paradermal and cross sections of 10 μm were made of the entire gland of each species. Sections were then mounted onto slides and stained with safranin and fast

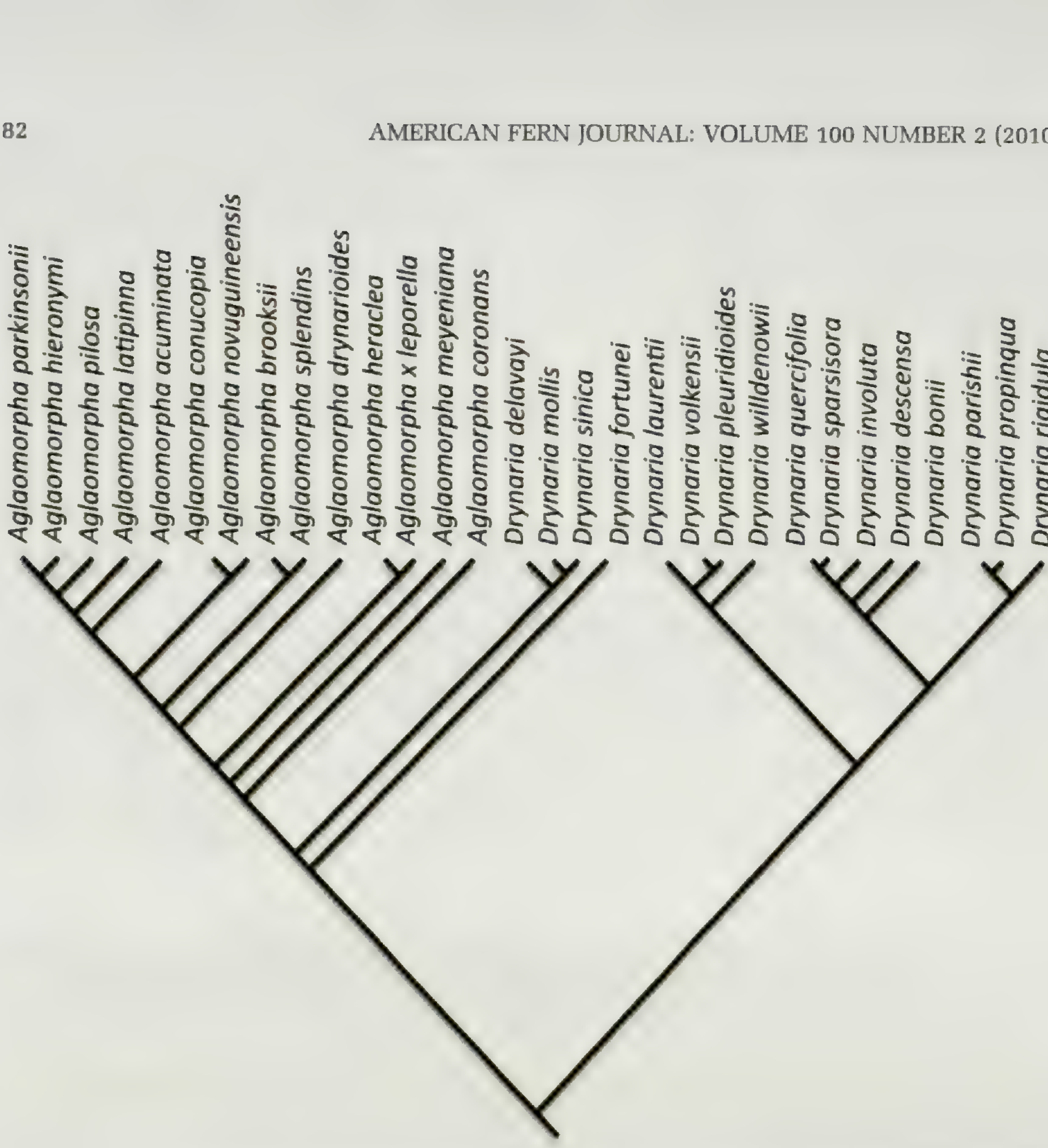
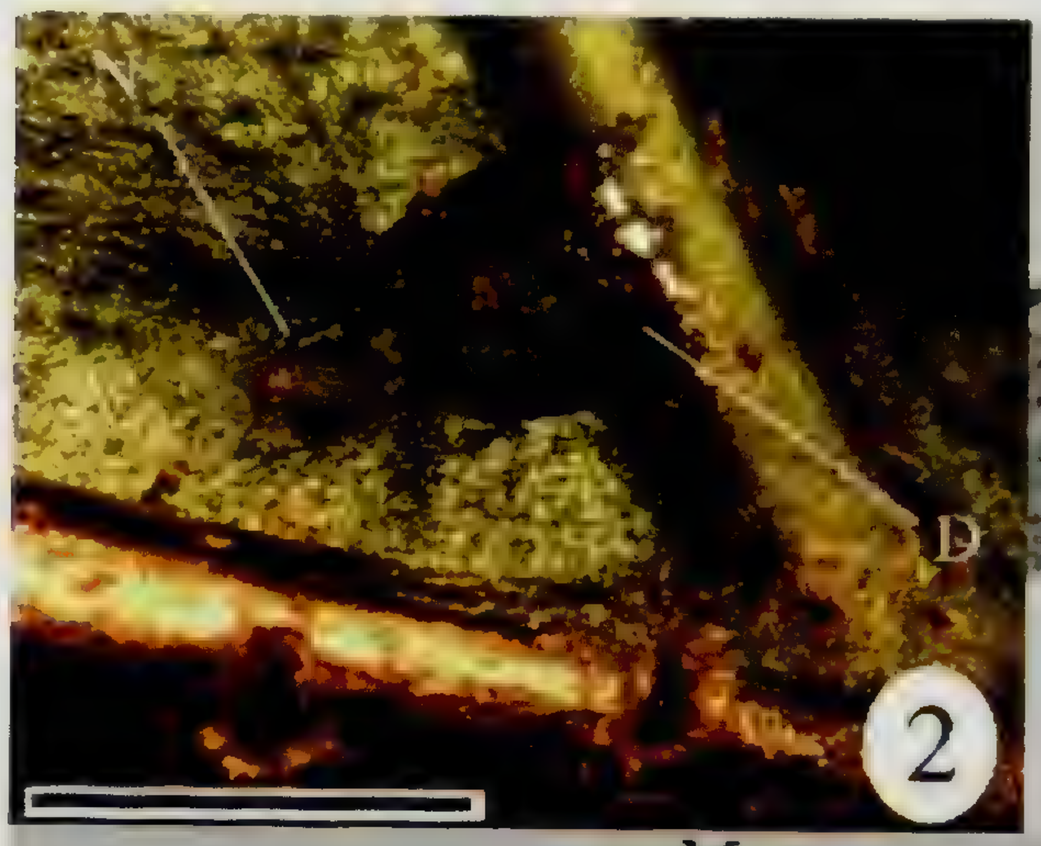


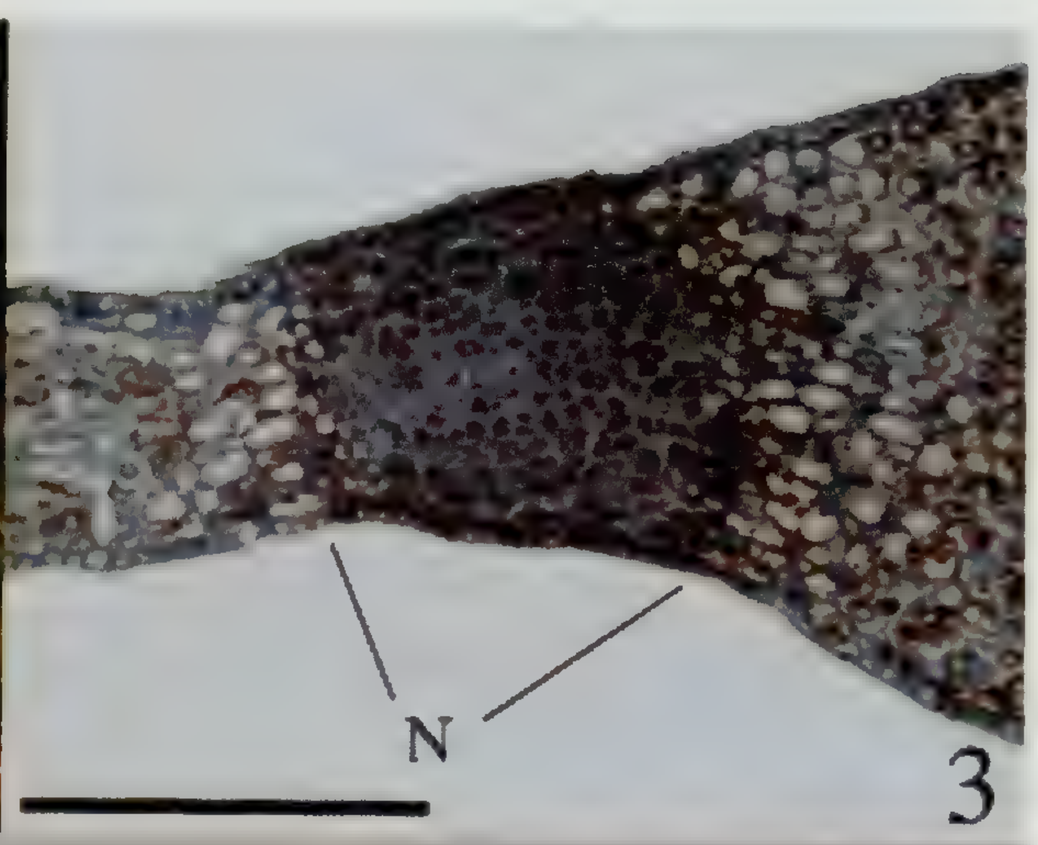
FIG. 1. Phylogenetic tree from (with permission) Janssen and Schneider (2005). Based on the strict consensus tree of the two most parsimonious trees with the combined data set (morphological and genetic).

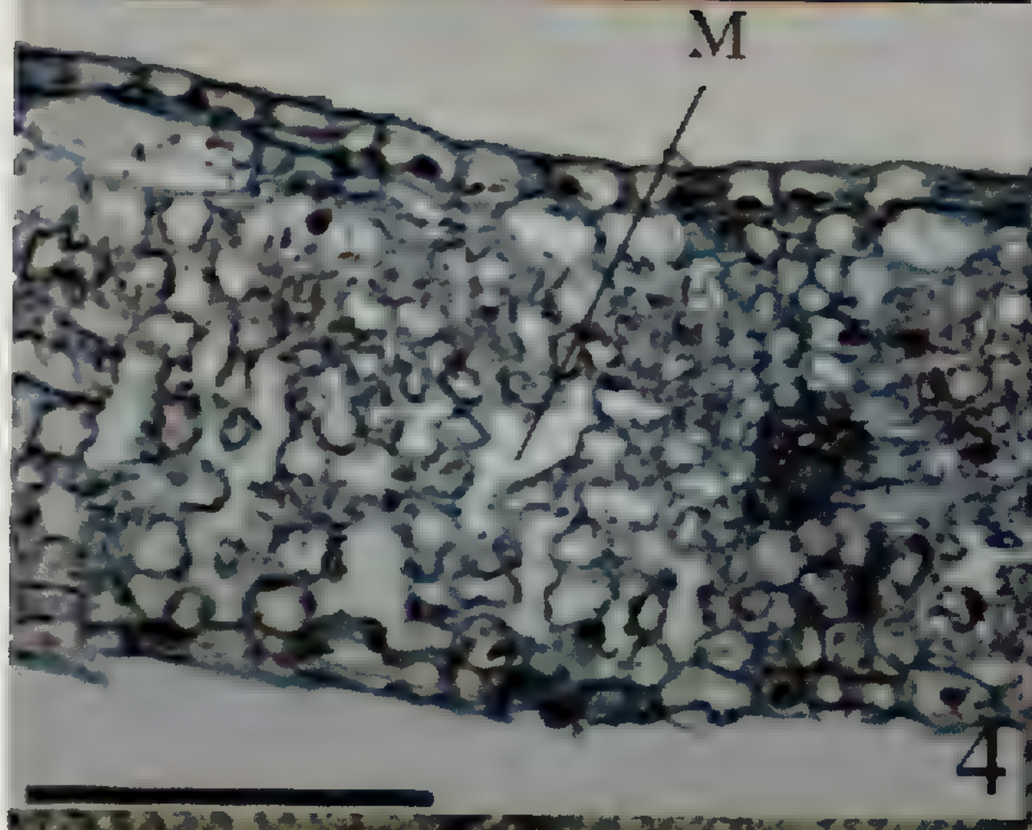
green. Samples of *A. coronans* were mature leaves and using the standard technique did not produce quality sections. Longitudinal hand-sections were made of these glands. All of the slides were examined using a standard light microscope and digital images were prepared and labeled using Adobe Photoshop. Measurements were made using a stage micrometer.

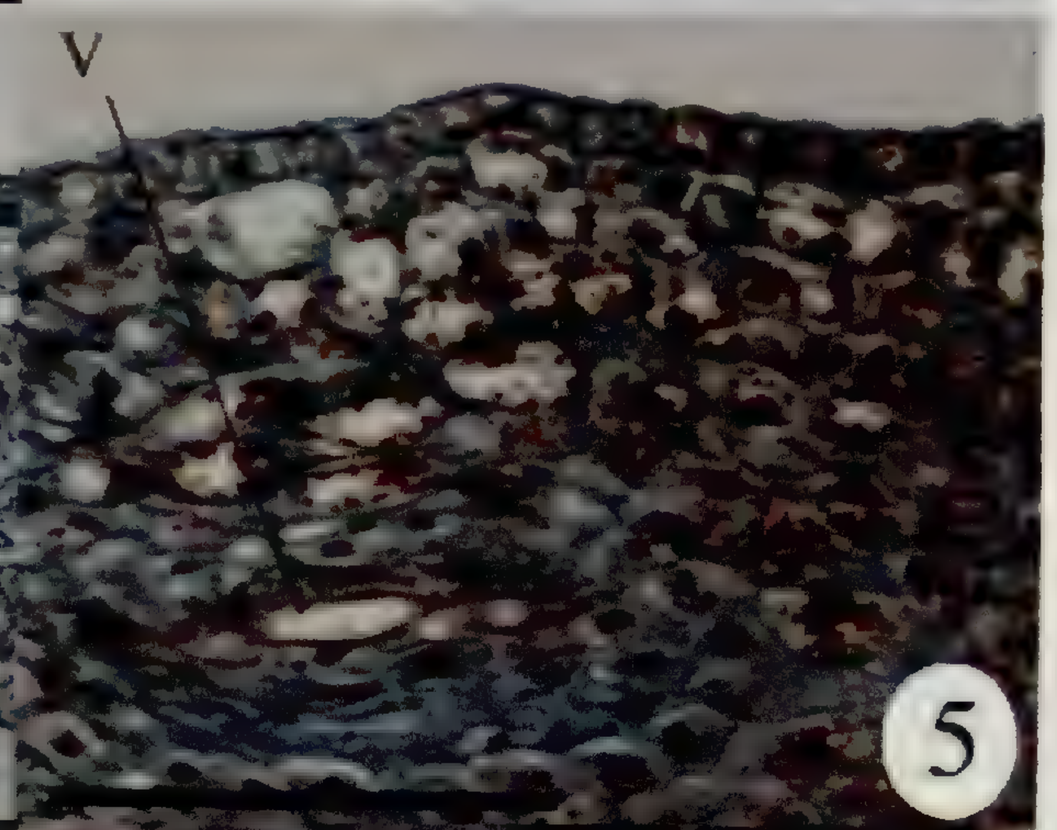
RESULTS

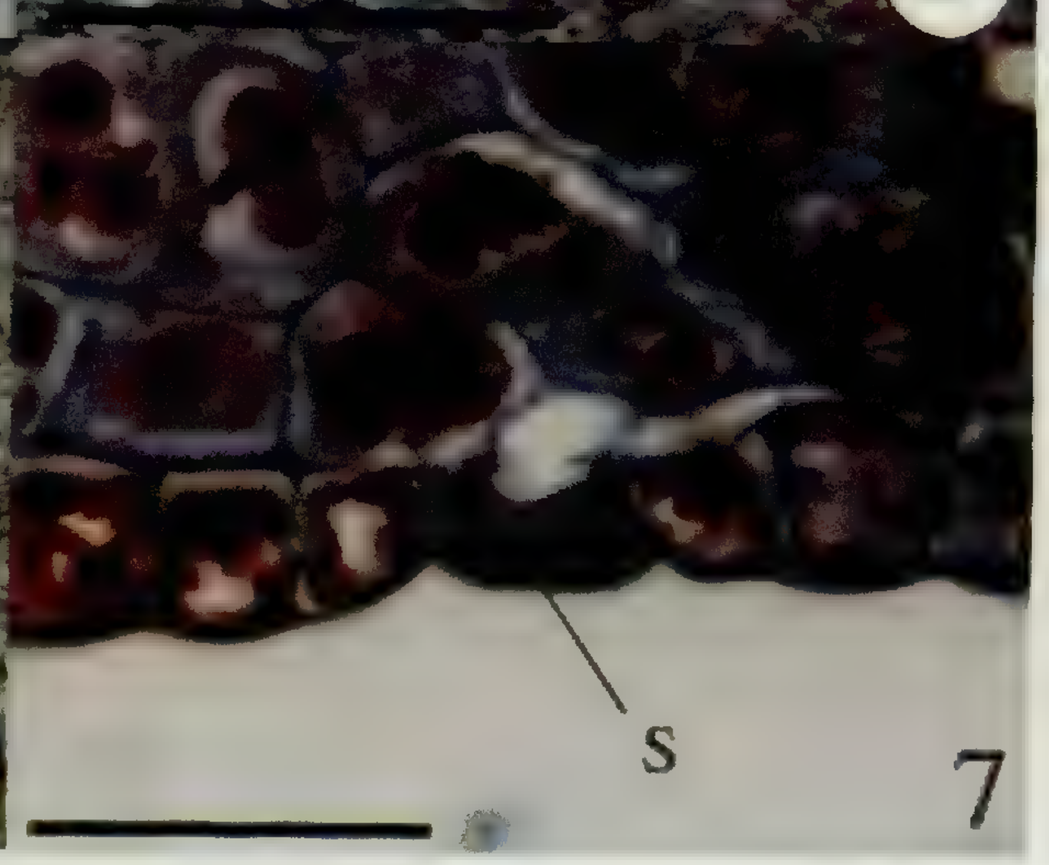
Drynaria quercifolia.—Nectaries are present on both foliage leaves and nest leaves. The glands are translucent patches approximately 1–2 mm in diameter. Clear liquid was observed on the abaxial surface of the glands (Fig. 2). On the foliage leaf they occur at the junction of lateral veins with the main vein, although a nectary does not occur at every junction. When they do occur, glands are present distal to the junction. The nectaries continue down the











FIGS. 2–7. *Drynaria quercifolia* foliar nectaries. 2. Nectary on abaxial surface of leaf. 3. Longitudinal section of nectary perpendicular to rachis. 4. Longitudinal section of leaf. 5. Longitudinal section of the nectary perpendicular to the rachis. 6. Paradermal section of gland. 7. Longitudinal section of stomata on abaxial surface of the nectary. Bar in 2 = 2 mm; bars in 3, 4, 6 = 5 μm; bar in 5 = 2 μm; bar in 7 = 0.5 μm; N = nectary; D = droplet; M = spongy mesophyll; S = stomata; V = vascular bundle.

petiole at regular intervals in the absence of an associated leaf lobe. In addition, smaller nectaries, less than 1 mm in diameter, occur on the leaf lamina with no regular pattern although they occasionally occur in clusters. The nest leaves also bear nectaries.

In cross-section, the nectary extends from the adaxial surface of the leaf to the abaxial surface. There is no variation in the thickness of the leaf although there are more layers of cells in the nectary than in the surrounding leaf blade (Fig. 3). The nectariferous tissue of *D. quercifolia* is distinct from surrounding leaf tissue (Fig. 4). The cells in the glandular region are darkly staining and approximately 0.4 μm smaller in diameter than the leaf lamina cells. Plastids in the glandular cells are non-green and the glandular region lacks prominent intercellular spaces (Figs. 5 and 6) whereas intercellular spaces are prominent in the leaf mesophyll (Fig. 4). Nectaries are bordered by specialized branches of vascular tissue (Figs. 5 and 6). These veins come into direct contact with the glandular region and contain both xylem and phloem. Stomates are present on the abaxial surface of the nectary (Fig. 7).

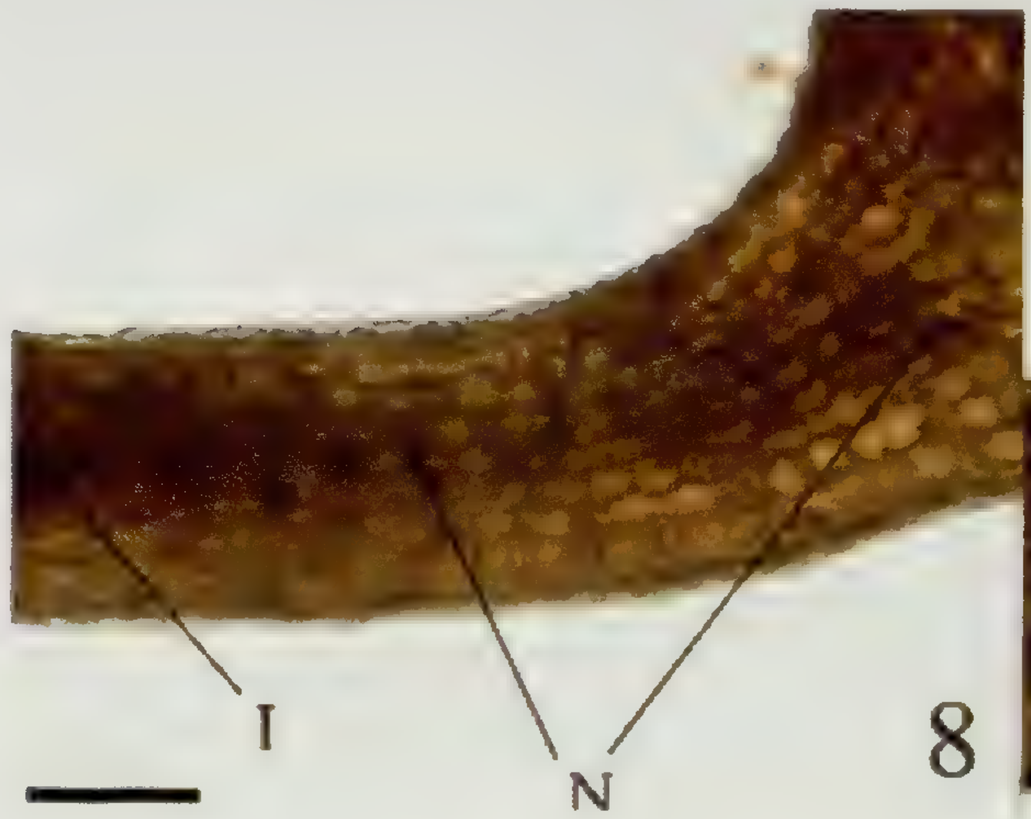
Aglaomorpha coronans.—Nectaries are approximately 2 mm in diameter, occur distal to the junction of the main vein with a lateral vein and are visible as translucent spots or patches in the abaxial surface of the leaf tissue. They are not present at every junction. Smaller nectaries, less than 1 mm in diameter, that are not associated with the main vein were observed on the leaf lamina. They occur individually and in groups, and are scattered throughout the leaf blade.

Hand sections of the larger nectaries (Fig. 8) reveal a glandular region of clear cells distinct from the surrounding leaf blade (Fig. 9). The glandular tissue lacks prominent intercellular spaces and its cells are approximately 0.1 μm smaller in diameter than those of the leaf blade.

The nectaries not associated with the main (Fig. 10) vein lack chlorophyll and prominent intercellular spaces. They are composed of cells approximately 0.1 μm smaller in diameter than cells of the leaf blade tissue (Fig. 11). They are also different from the larger glands because they are composed of the same number of cell layers as the surrounding unmodified leaf tissue. As a result, the thickness of the leaf at the gland is less than that of the surrounding non-glandular region.

Drynaria rigidula.—Nectaries of this species occur on the basiscopic side of leaflets. They are concavities on the abaxial surface of the leaflet base with an approximate diameter of 0.5–1 mm. The depression is formed by the midvein on one side of the nectary and a secondary vein on the opposite side. Clear liquid was observed on the abaxial surface of most nectaries and glands appeared to be more active on the basal half of fronds. Dark-colored mold was often observed on the nectaries (Fig. 12). Similar nectaries occur on the petiole of the frond in the absence of leaflets.

The nectariferous cells (Figs. 13 and 14) are approximately 0.03 μm smaller in diameter than those of the leaf (Fig. 15). Unlike this surrounding leaf blade tissue, the glandular cells lack prominent intercellular spaces and plastids in the nectary are non-green (Fig. 14). Vascular tissue does not branch into the



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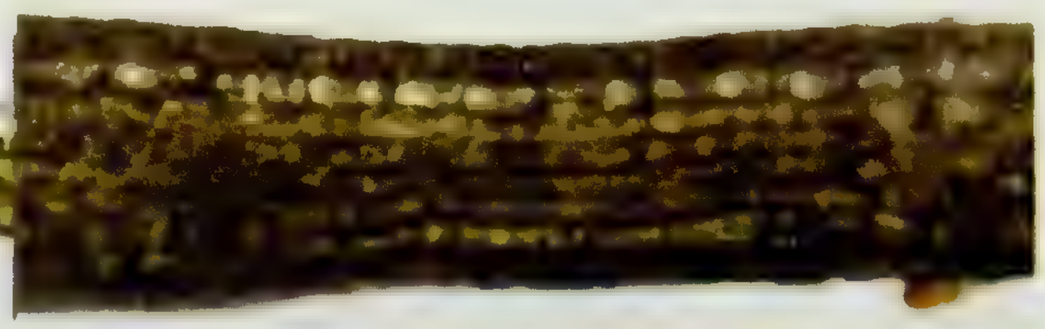

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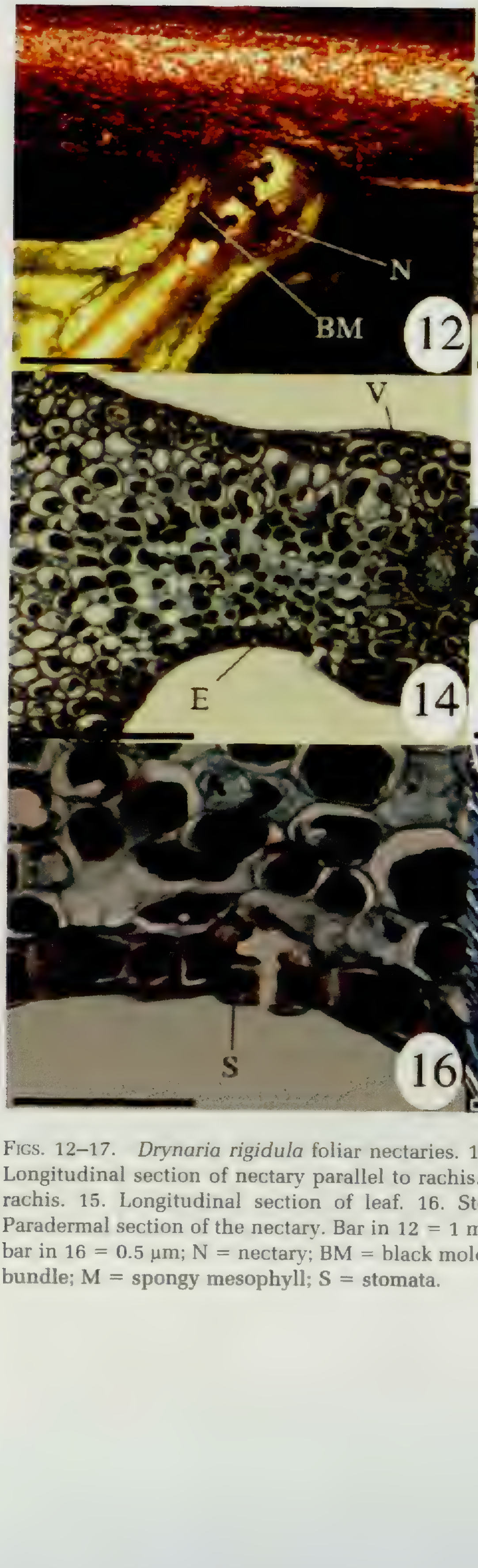
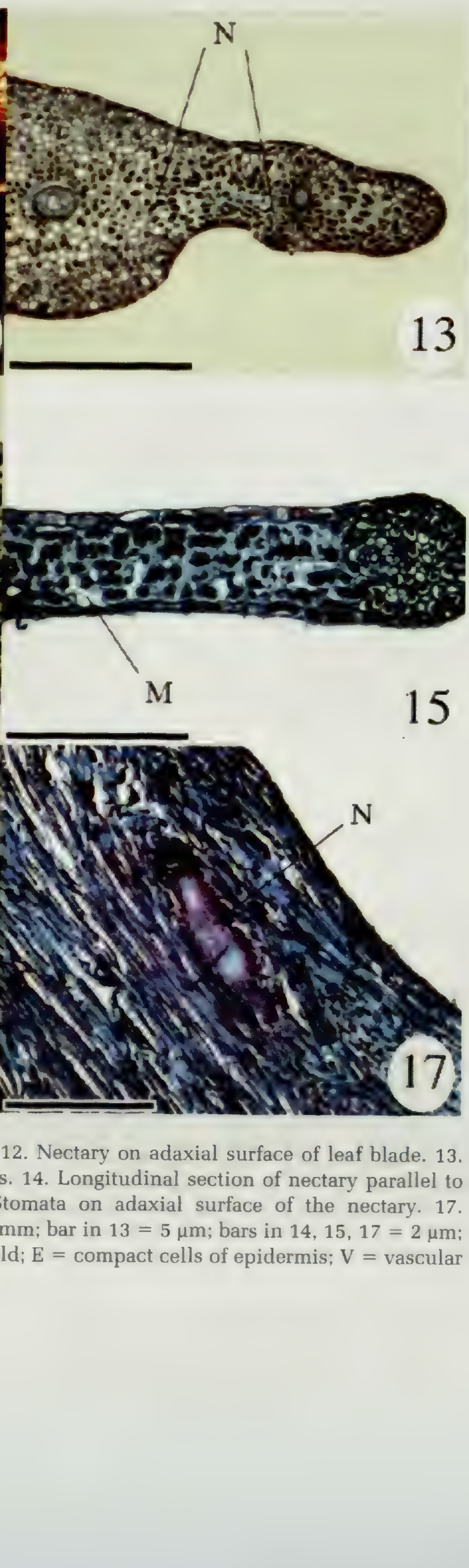
FIGS. 8–11. *Aglaomorpha coronans* foliar nectaries. 8. Longitudinal hand and section of nectary. 9. Longitudinal hand section of foliage leaf. 10. Longitudinal hand section of smaller nectary on leaf blade. 11. Longitudinal hand section of leaf. Bars in 8, 9, 11 = 2 μm; bar in 10 = 5 μm; N = nectary; I = intercellular spaces.

gland but its rim is formed by a secondary vein (Fig. 14). This vascular tissue consists of both xylem and phloem. Stomates occur on the abaxial surface of the nectary (Fig. 16). In paradermal section, the nectary is apparent from differences in the tissue staining (Fig. 17).

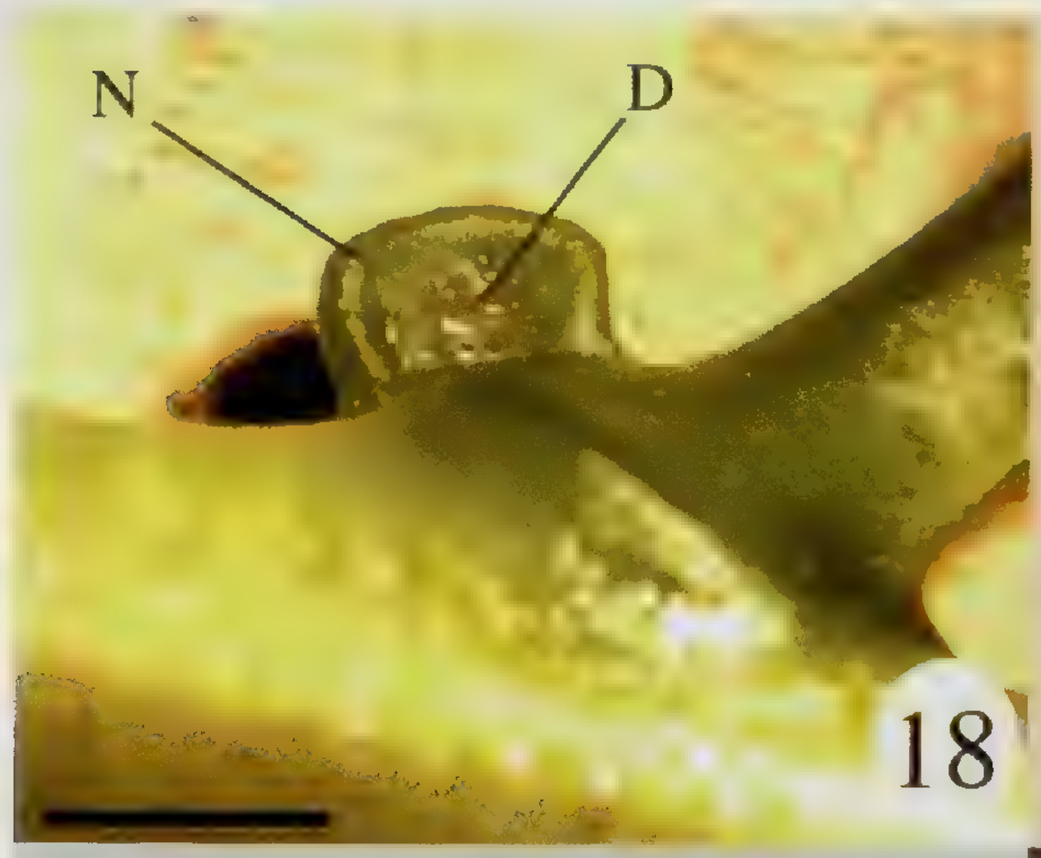
Aglaomorpha acuminata.—This species bears nectaries on specialized concave structures that are separate from the leaf blade as stand-alone structures. They are approximately 3 mm in diameter and occur at the base of each leaflet on the basiscopic side of the leaflet's junction with the rachis (Fig. 18). Less developed nectaries extend down the petiole even in the absence of leaflets. Clear liquid was observed on the abaxial surface of the nectaries (Fig. 18).

The anatomy of the nectary is complex in its composition and staining. Vertical sections show cellular zonation with a heavier staining glandular region surrounded by lighter staining non-glandular tissue (Fig. 19). Distinct from the leaf blade tissue (Fig. 20), the glandular cells are concentrated near the center of the protruding structure and non-glandular tissue makes up the





FIGS. 12–17. *Drynaria rigidula* foliar nectaries. 12. Nectary on adaxial surface of leaf blade. 13. Longitudinal section of nectary parallel to rachis. 14. Longitudinal section of nectary parallel to rachis. 15. Longitudinal section of leaf. 16. Stomata on adaxial surface of the nectary. 17. Paradermal section of the nectary. Bar in 12 = 1 mm; bar in 13 = 5 μm; bars in 14, 15, 17 = 2 μm; bar in 16 = 0.5 μm; N = nectary; BM = black mold; E = compact cells of epidermis; V = vascular bundle; M = spongy mesophyll; S = stomata.



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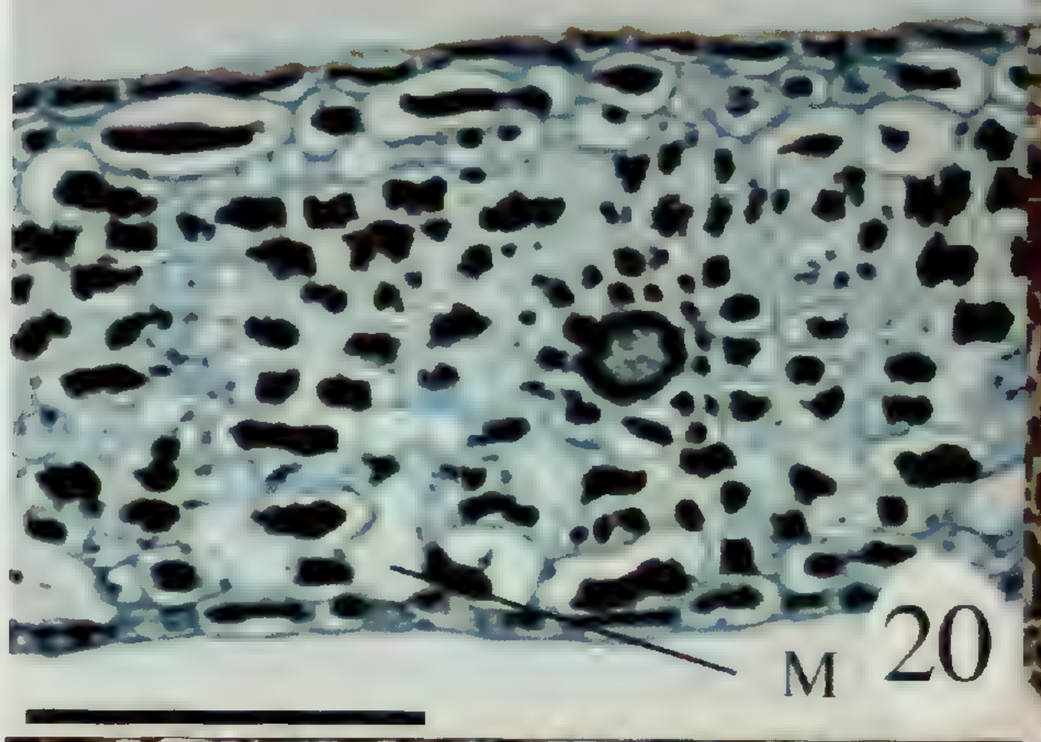

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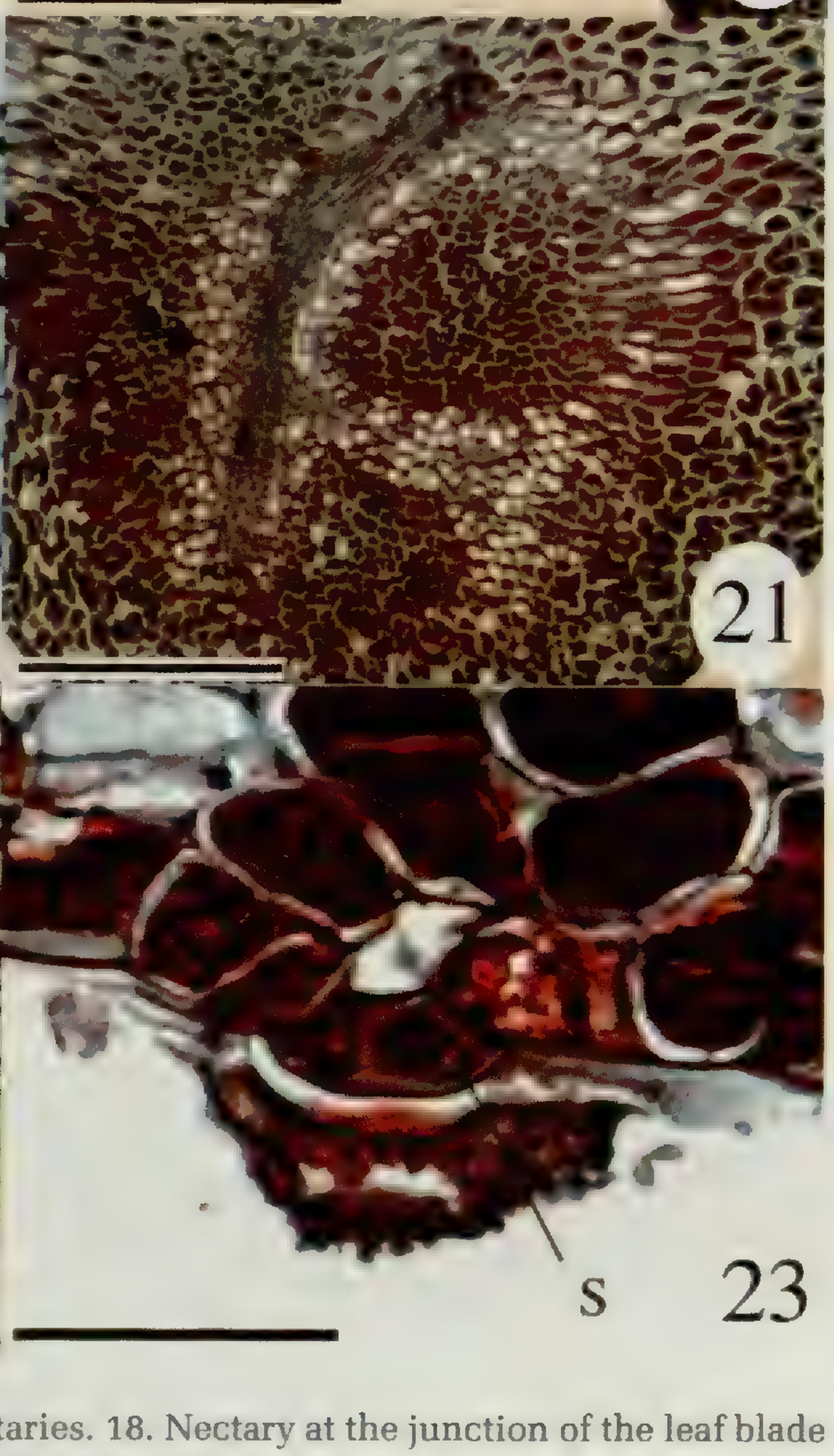

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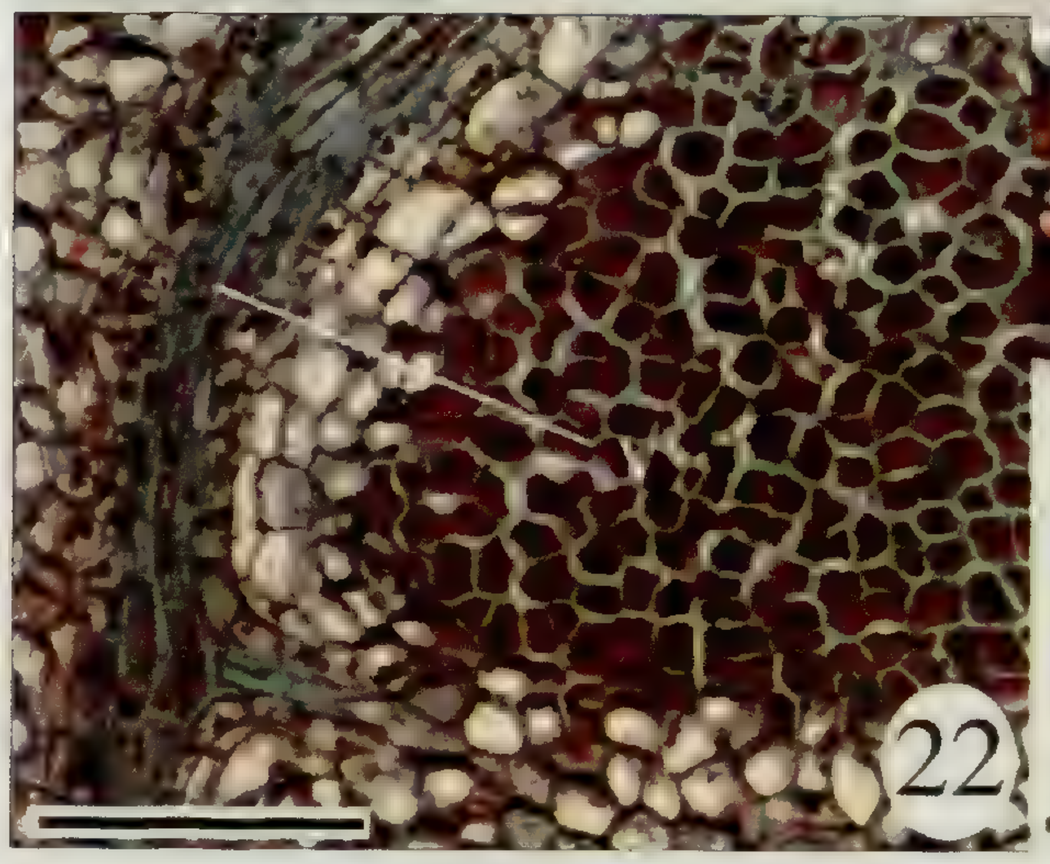

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FIGS. 18–23. *Aglaomorpha acuminata* foliar nectaries. 18. Nectary at the junction of the leaf blade to the rachis. 19. Longitudinal section of the gland perpendicular to the rachis. 20. Longitudinal section of the leaf. 21. Paradermal section of the nectary. 22. Paradermal section of the nectary. 23. Stomata and mold on the nectary. Bar in 18 = 3 mm; bars in 19, 21 = 5 µm; Bars in 20, 22 = 2 µm; Bar in 23 = 0.5 µm; D = droplet; N = nectary; V = vascular bundle; M = spongy mesophyll; S = stomata.

rim (Fig. 21). The leaf blade is composed of cells with green plastids and prominent intercellular spaces. Glandular region cells are approximately 0.18 μm smaller in diameter than those of standard leaf blade with non-green plastids. The tissue lacks prominent intercellular spaces. Vascular tissue branches within the nectary and is closely associated with the darkest staining tissue. In paradermal sections, the vein encircles the darker staining region and it is immediately surrounded by lighter staining cells (Figs. 21 and 22). Xylem and phloem are both present in the vascular bundle (Fig. 22). Stomates occur on the abaxial surface of the nectary (Fig. 23).

Discussion

In most plants with nectaries, these structures are comprised of tissue that is small-celled, thin-walled and densely staining with reduced intercellular spaces compared to the surrounding tissue. These cells are often surrounded by sub-glandular tissue and by modified epidermal cells with a thick cuticle. The secretion of nectar may occur either through stomata on the epidermis of the nectariferous tissue or through epidermal cells or trichomes (Fahn, 1979; Elias, 1983).

Nectaries vary in their degree of vascularization according to taxa. These veins differ both in their proximity to the gland and in the composition of their tissue. Veins, when they occur, may consist of both xylem and phloem or only one of these. Often vascular tissue does not come into direct contact with the nectariferous tissue and ends in the subglandular cells. In the nectaries of other plants, vascular tissue may be entirely absent (Durkee, 1983; Elias, 1983). This specialization and variability in nectary vascularization, however, is often associated more with the size of the nectary than with its systematic specialization (Elias, 1983).

The nectaries of *Drynaria quercifolia*, *D. rigidula*, *Aglaomorpha acuminata* and *A. coronans* are anatomically similar in many features. They are vascularized glands composed of a region of densely staining cells that are smaller and more isodiametric than the surrounding non-glandular tissue. The nectariferous tissues have minute or absent intercellular spaces and vascular tissue is closely associated with the nectaries. This anatomical study is the first that has documented such a close association between nectaries and vascular tissue in ferns. All of the nectaries secrete nectar and have stomates in their abaxial epidermis.

Differences in the anatomy and distribution of the observed drynarioid nectaries are notable (Table 1). The nectaries occurring on *Drynaria quercifolia* and *A. coronans* are more similar to one another than to those of the other two species. *Drynaria quercifolia* and *A. coronans* nectaries occur as patches abutted by non-glandular leaf blade tissue and they are located mainly distal to the vein junction. Glands have also been observed basal to the veins (Zamora and Vargas, 1974; Chandra, 1980; Koptur *et al.*, 1982). In contrast, the glands of *D. rigidula* and *A. acuminata* are stand-alone structures separate from the leaf blade, occur consistently on and are restricted to the basiscopic side of the leaflet base. They continue down the petiole in absence of leaflets.

TABLE 1. Summary of results and nectary type classification.

Species	Anatomy	Vascular tissue	Distribution
Drynaria quercifolia	Patch of densely staining cells	Abutting the nectary	Inconsistent. Mostly distal to the midvein-primary vein junction
Aglaomorpha coronans	Patch of cells	Abutting the nectary	Inconsistent. Mostly distal to the midvein-primary vein junction
Drynaria rigidula	Small concavity of densely staining cells	Forming the rim of the nectary	Restricted to the basiscopic side of the petiole
Aglaomorpha acuminata	Large spoon-like appendage of densely staining cells	Branching within the nectary	Restricted to the basiscopic side of the petiole

Furthermore, the nectaries of *D. rigidula* are distinct in their reduction in size and *A. acuminata* nectaries are distinct in their large size (see scale bars in *Results* for size). The *A. acuminata* nectary is uniquely elaborate in comparison to any known fern nectary and is more similar to some angiosperm nectaries (Elias, 1983; Thadeo *et al.*, 2008, Zimmerman, 1932).

Despite similar pinnate leaf dissection and gland distribution, *D. rigidula* and *A. acuminata* occur on different branches of the cladogram (Fig. 24) (Janssen and Schneider, 2005). Therefore, these nectaries appear to be independently derived. Although *A. acuminata* nectaries are much more complex than those of the other drynarioid species in this study, further anatomical studies of other *Aglaomorpha* species will clarify the extent to which this complexity is shared within the genus.

Nectariferous patches, like those observed in *D. quercifolia* and *A. coronans*, are common throughout drynarioid ferns. The patches have also been observed in *A. heraclea*, *A. meyeniana*, *A. splendens* and *D. descensa* (Fig. 24) (Zamora and Vargas, 1974; pers. comm. Turner, 2006). *Drynaria rigidula* and *A. acuminata* are the only species, out of the four studied, with distributionally-restricted and stand-alone structures. Nectaries occur on various other species of ferns (Koptur, Smith and Baker, 1982), although relatively few anatomical studies of these glands have been conducted. Bracken (*Pteridium*) (Page, 1982; Power and Skog, 1987; Rumpf *et al.*, 1994) and *Cyathea* (pers. comm. White, 2007) are two examples of ferns with nectaries. The anatomy, ultrastructure and physiology of *Pteridium* nectaries have been described in detail. Similar investigations of the structure and function of drynarioid nectaries could be valuable.

Pteridium nectaries have been documented in great detail and are different from drynarioid nectaries in both distribution and anatomy. Bracken glands occur on the leaf axis (at the junctions of the stipe and midribs of the pinnae and also at the junctions of the midribs of the pinnae and the midribs of the pinnules). Drynarioid nectaries, in contrast, occur either on expanded leaf blades or on structures that remain on the petiole after foliar tissue has been reduced to form pinnate leaves. Anatomically, *Pteridium* nectaries differ from those of drynarioid ferns in that 1) vascular tissue does not form specialized

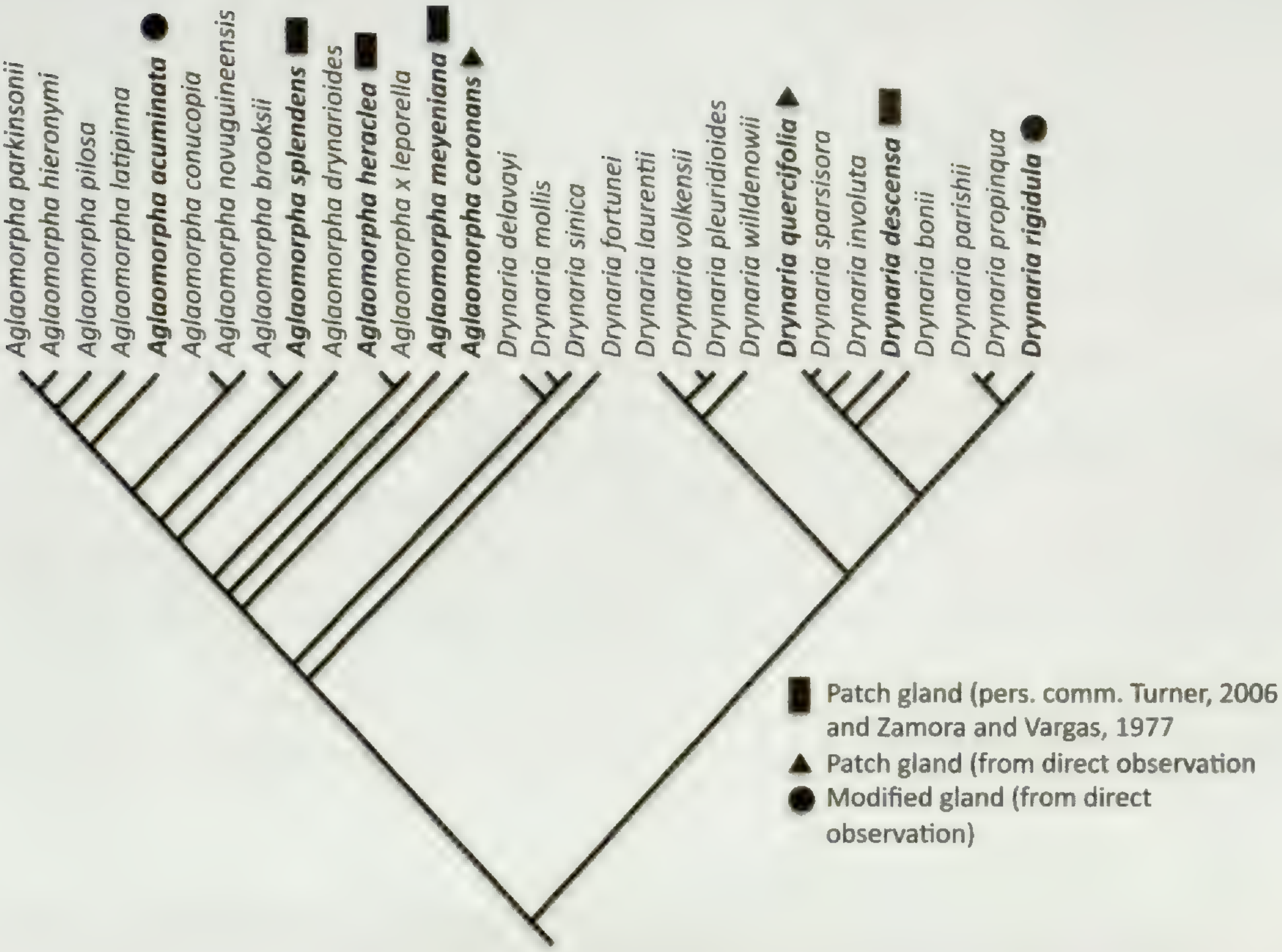

FIG. 24. Presence of nectaries on Janssen and Schneider's (2005) phylogenetic tree (used with permission).

branches abutting or extending into the nectary, 2) stomata are present in a greater abundance on the surface of the nectary, and 3) the nectariferous tissue has glandular and subglandular layers (Power and Skog, 1987; Rumpf *et al.*, 1994). Drynarioid glands are relatively simple in structure. Only in *A. acuminata* was zonation observed.

There are, however, some basic similarities between the nectaries of *Pteridium* and drynarioid ferns. Both nectaries are visible macroscopically and thus belong to the "structural" type as of extrafloral nectary (Zimmerman, 1932). They are both composed of tissue that is distinct from the surrounding parenchyma. As in the drynarioid ferns, the nectariferous cells of *Pteridium* are small, isodiametric, densely staining and associated with stomates (Power and Skog, 1987). Another similarity between the glands of the two groups is the decrease in secretory activity as the gland ages and matures (Power and Skog, 1987; Rumpf *et al.*, 1994). Although this study did not examine the anatomy of nectaries at different ages, active nectaries were absent on older portions of fronds.

CONCLUSIONS

Detailed anatomical data help clarify phylogenetic analyses (Elias, 1983; Stuessy and Crisci, 1984), and this purpose is one possible application for the

anatomical differences and similarities of drynarioid fern nectaries. The most recent study of the two genera combines morphological and molecular data to conclude that *Drynaria* is paraphyletic and *Aglaomorpha* is monophyletic (Fig. 1) (Janssen and Schneider, 2005). However, the morphological data set *alone* contradicts the molecular evidence and the study does not reject the null hypothesis of a monophyletic *Drynaria*. Adding an anatomical analysis of drynarioid nectaries throughout the genera may further clarify the phylogeny of *Drynaria*.

Although the nectaries of only four drynarioid species were examined, the data contribute significantly to the anatomical knowledge of these glands. The presence of vascularized nectaries composed of cells lacking prominent intracellular space was observed. The glands are distinct from the surrounding tissue and the similarity in anatomy and distribution of the nectaries of *D. rigidula* and *A. acuminata* may be independently derived. Contrary to the only previous anatomical description, drynarioid fern nectaries are not composed of columnar cells and although they are vascularized, they are not associated with a central vascular plexus, as reported (Nayar, 1961). The *Aglaomorpha acuminata* nectary is unique among known ferns in its anatomical complexity. Future studies should consider surveying other taxa, such as *Pleopeltis*, and implementing different staining techniques.

Acknowledgments

Thank you to Dr. Richard White for his guidance and patience, to Melvin Turner, who unselfishly lent his time and invaluable expertise and to our reviewers for their insightful comments.

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American Fern Journal 100(2):93–102 (2010)

Ultrastructural Observations of Oogenesis in the Fern *Adiantum flabellulatum* L. (Adiantaceae)

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Abstract.—Oogenesis in the fern *Adiantum flabellulatum* was studied using transmission electron microscopy. The newly formed egg is closely appressed to the archegonial jacket cells. Plasmodesmata connect the egg and the ventral canal cell, but are absent between the egg and the jacket cells. During maturation, a separation cavity forms around the egg. However, a pore region, persistently connecting the egg and the ventral canal cell, is reported for the first time. Subsequently, an osmiophilic egg envelope is formed, from a combination of the products of sheets of endoplamic reticulum and osmiophilic bodies in the egg cytoplasm. The absence of this envelope from the region where the egg and ventral canal cell were formerly interconnected produces a fertilization pore, which is believed to be an entrance for the sperm penetrating the egg. In the mid stages of egg maturation, the nucleus envelope produces extensive evaginations which then appear to be sequestered into the egg cytoplasm recalling a similar phenomenon in the eggs of other ferns. Degeneration of the neck canal cells is associated with the secretion of copious mucilaginous material.

Key Words.—*Adiantum flabellulatum*, fern, fertilization pore, nuclear evagination, oogenesis

Previous studies on oogenesis in ferns including *Pteridium aquilinum* (L.) Kuhn (Bell and Mühlethaler, 1962a, b; Bell and Duckett, 1976), *Histiopteris incisa* (Thunb.) J.Sm. (Bell, 1980), *Osmunda cinnamomea* var. *asiatica* Fern. (Bao *et al.*, 2003), and *Dryopteris crassirhizoma* Nakai (Bao *et al.*, 2005) have revealed that the egg is surrounded by a conspicuous extra egg 'membrane'. This structure has been regarded as a venter coat covering the top of the egg in *Athyrium filix-femina* (L.) Roth (Fasciati *et al.*, 1994) and *Ceratopteris richardii* Brongn. (Lopez-Smith and Renzaglia, 2008). In addition derived taxa such as *Pteridium aquilinim* (Bell, 1972), *Histiopteris incisa* (Bell, 1980), *Dryopteris crassirhizoma* (Bao *et al.*, 2005) and *Athyrium filix-femina* (Fasciati *et al.*, 1994) produce conspicuous nuclear evaginations during egg maturation. In contrast, this feature appears to be absent in more primitive families (e.g., Marattiaceae, Osmundaceae, Schizaeaceae and Dipteridaceae (Bell, 1986; Bao *et al.*, 2003)). We recently discovered in *Ceratopteris thalictroides* (L.) Brongn. that the extra egg membrane is absent from the central part of the upper egg surface and that this acts as a fertilization pore, through which the spermatozoid penetrates the egg (Cao *et al.*, 2009). This structure in the egg of a sister species, *Ceratopteris richardii*, has been interpreted as a receptive

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site (Lopez-Smith and Renzaglia, 2008). No detailed description about the formation of the structure has been reported; however, whether the fertilization pore exists in other ferns has not been investigated. Here we describe the development of a fertilization pore in oogenesis in *Adiantum flabellulatum* L. (Adiantaceae) and detail other noteworthy features of oogenesis in this fern.

Materials and Methods

Spores of *Adiantum flabellulatum* were collected from plants in Wanmulin Nature Reserve of Fujian province, China. The spores were surface sterilized with 5% sodium hypochlorite solution for 3 min. After rinsing three times with distilled water, the spores were sown on a modified Knop's solution (0.8g $Ca(NO_3)_2 \cdot 4H_2O$; 0.2g KH_2PO_4; 0.2g KNO_3; 0.2g $MgSO_4 \cdot 7H_2O$, dissolved in 1 liter distilled water), solidified with 1.5% agar in culture dishes. These dishes were placed in an artificial climate chamber under conditions of 25°C in the light (18 h) and 20°C in the dark (6 h). After 6 to 7 weeks, archegonia had developed on the lower surface of the gametophytes just behind the growing apex.

Gametophytes bearing archegonia were placed in 3% glutaraldehyde in 0.1 mol/L phosphate buffer at room temperature for 6–12 h. The specimens were subsequently washed three times with the same buffer, postfixed in 2% aqueous osmium tetroxide for 2 h, rinsed three times in buffer and embedded in Spurr's resin (SPI-Chem, USA) via a graded acetone series. Specimens were thick sectioned for the presence of the archegonia and thin-sectioned with a diamond knife on an Ultracut-E ultramicrotome (Reichert-Jung, Germany). The thin sections were stained with uranyl acetate and lead citrate. All specimens were observed with H-600 electron microscope (Hitachi, Japan).

Results

The newly formed egg.—The archegonium of *A. flabellulatum* contains three cells; the egg, the ventral canal cell (VCC), and the neck canal cell (NCC) (Fig. 1A). When the egg is newly formed, the periphery of the three cells is closely appressed to the archegonial jacket cells (Fig. 1A–D). There are numerous plasmodesmata between the egg and the VCC, and also between the VCC and NCC (Fig. 1D, E) but these are absent between the three cells and the jacket cells (Fig. 1C, D). In longitudinal sections the young egg is approximately trapezoidal or quadrate (Fig. 1A, B). The transverse diameter of the young egg is about 25μm and its height is about 15–17μm (Fig. 1A, B). The nucleus of the young egg is roughly spherical and typical sections show one or two nucleoli (Fig. 1B). The chromatin of the egg cell is slightly more dispersed than that in the VCC (Fig. 1B). The newly formed egg contains abundant vesicles, distributed principally around its periphery and base (Fig. 1B) and plastids with large starch grains and few lamellae (Fig. 1C). Mitochondria are distributed evenly throughout the cytoplasm of the egg (Fig. 1B, C). At this

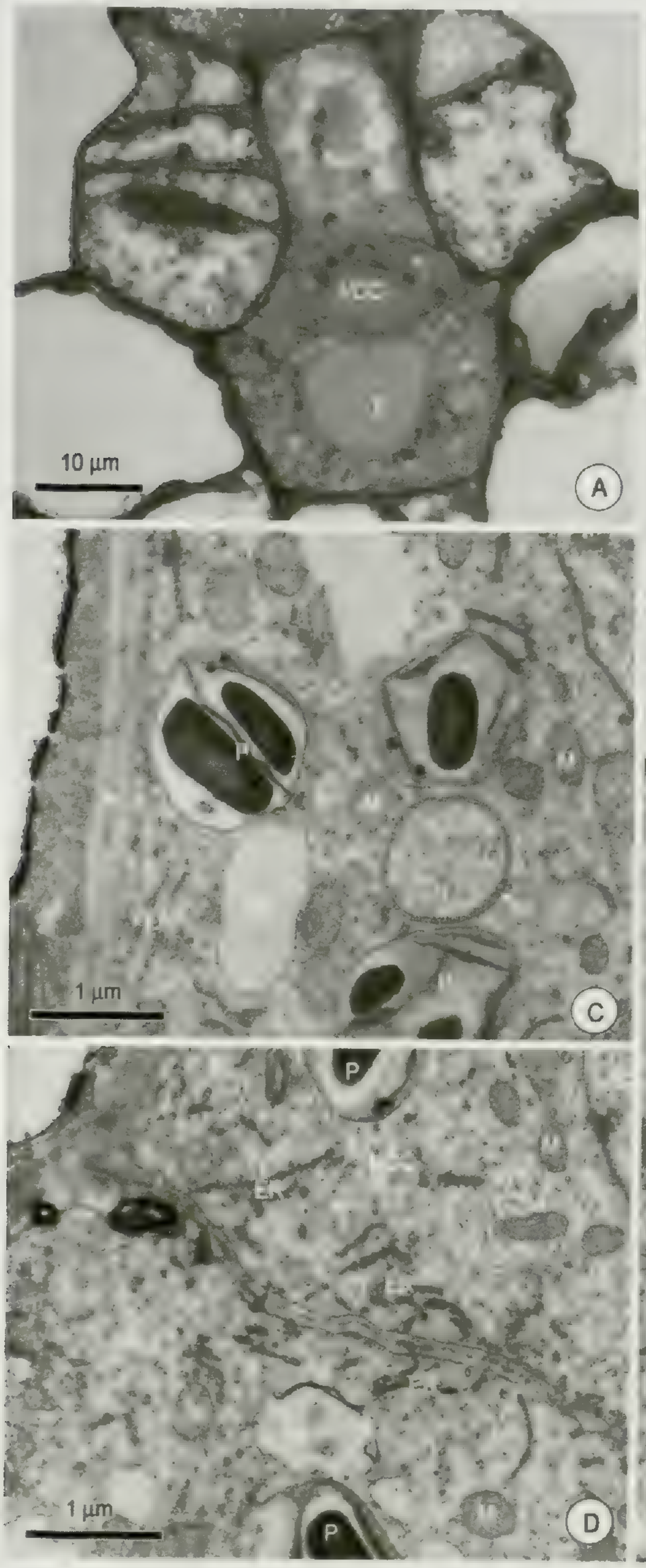


FIG. 1. Young archegonium of *Adiantum flabellulatum*. **A.** An archegonium, containing a young egg (E), a ventral canal cell (VCC) and a neck canal cell (NCC) (under LM, all other figures are observed under TEM). **B.** An archegonium, containing a young egg (E), a VCC and a NCC. Arrow indicates a dumbbell-shaped mitochondrion in the VCC. **C.** Magnification of Fig. 1B, showing the organelles in the egg cell. Arrow indicates a sac-like mitochondrion. **D.** Part of the VCC and NCC showing the organelles. **E.** Plasmodesmata (Pd) connecting the egg and the VCC. A dumbbell-shaped mitochondrion (Md) lies beside the nucleus of the VCC. ER, endoplasmic reticulum; G, Golgi bodies; M, mitochondrion; N, Nucleus; Nus, nucleolus; P, plastid.

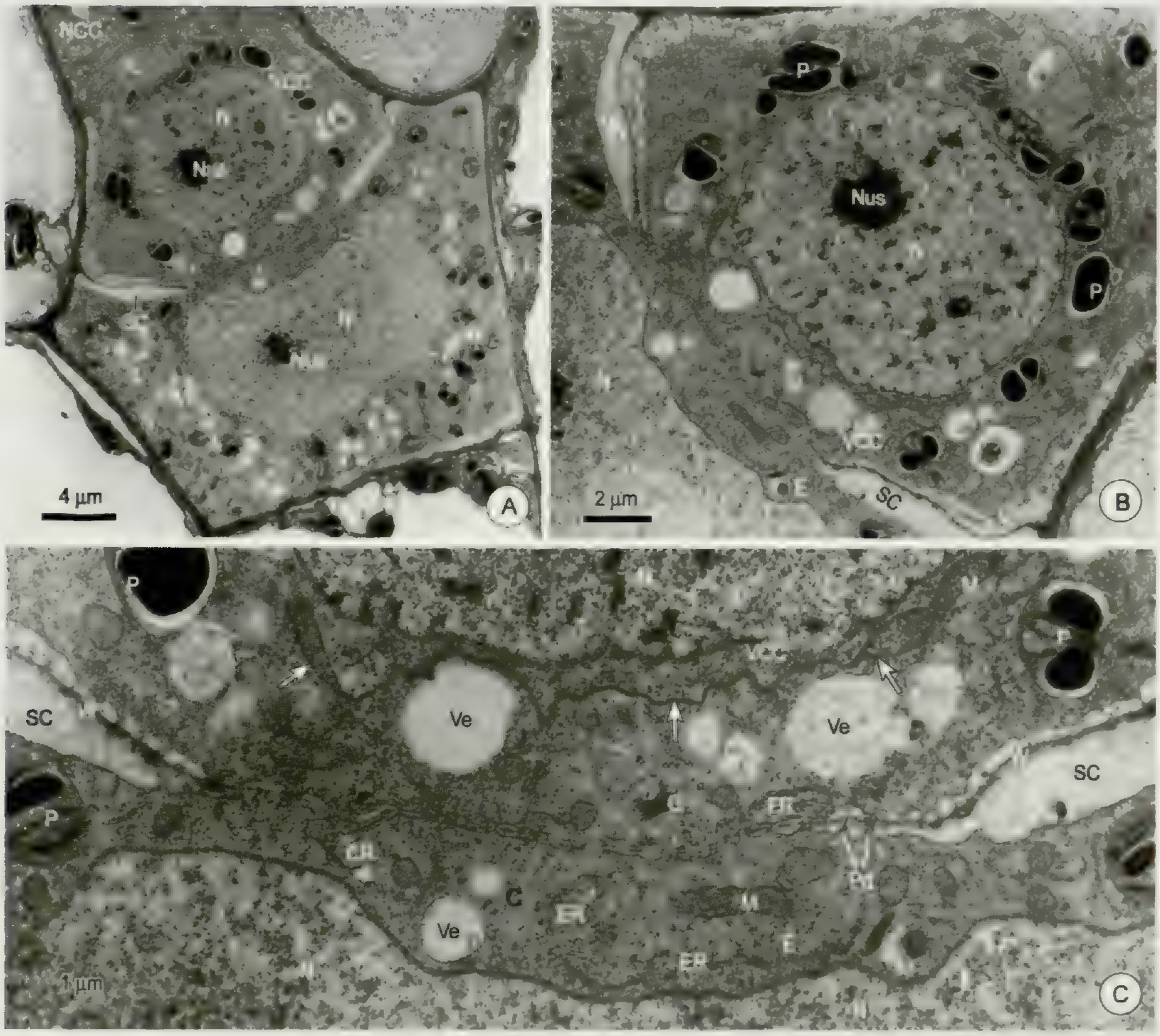


FIG. 2. Separation cavity formation. **A.** An archegonium, containing a maturing egg (E), a VCC and a NCC. A separation cavity (SC) has formed around the periphery of the egg. **B.** The VCC, showing the asymmetrical distribution of the organelles. **C.** Central region between the egg and the VCC, showing plasmodesmata (Pd) that connect the egg and the VCC; a sheet-like mitochondrion (arrows) lies beneath the nucleus of the VCC. E, Egg; ER, endoplasmic reticulum; G, Golgi body; M, mitochondria; N, Nucleus; Nus, nucleolus. P, plastid; Ve, vesicles; W, cell wall.

early stage the cytological features of the VCC, the NCC and the egg are closely similar (Fig. 1D) apart from the occurrence of dumbbell-shaped mitochondria in the lower lateral parts of the VCC (Fig. 1B, arrow; Fig. 1E).

Egg maturation.—Egg maturation is associated with substantial cytological changes including the formation of a separation cavity, an osmiophilic egg envelope, a fertilization pore and nuclear evaginations.

Formation of the separation cavity.—The separation cavity initially begins to form around the periphery of the upper surface of the egg and then extends around the lower part of the egg (Fig. 2A–C). However, the egg remains connected to the VCC by well- developed plasmodesmata at the central region in its upper surface (Fig. 2C). As the egg develops, the separation cavity above the egg expands centripetally and the connection region is correspondingly

reduced to a final diameter of about 2.7 μm (Fig. 3A, D). Plasmodesmata remain in the pore region (Fig. 3D). The nucleus now becomes somewhat irregular in outline with a depressed upper surface (Fig. 3A).

At this stage, the eggs contain single sheets of endoplasmic reticula (ER) often lying parallel to the plasmalemma (Fig. 3C). The egg plasmalemma becomes increasingly dark-stained except in the pore region (Fig. 3D) and dentate around the periphery (Fig. 3C, arrowhead). Vesicles containing osmiophilic materials are often present beneath the pore region (Fig. 3D) and in the cytoplasm of the VCC (Fig. 3B, arrows).

Organelles in the VCC are typically asymmetrically distributed. Plastids are clustered around the nucleus and are absent from the lower part of the cell (Fig. 2B), which contains vesicles, mitochondria and Golgi bodies (Fig. 2B, C; Fig. 3D). A large sheet-like mitochondrion lies beneath the nucleus of the VCC (Fig. 2E; Fig. 3D).

Formation of the osmiophilic egg envelope and the fertilization pore.—The second conspicuous feature of the maturing egg is the formation of a layer of osmiophilic egg envelope (formerly called 'extra egg membrane') around the egg (Fig. 4A). At this time parallel sheets of ER appear next to the lower surface of the egg (Fig. 4B), osmiophilic globules are visible next to the egg envelope (Fig. 4C, arrows), and the upper egg envelope is thicker than the lower part (Fig. 4B, C). The pore region still connects the egg cell with the VCC (Fig. 4A, D) and no osmiophilic egg envelope is formed in this region (Fig. 4D).

The organelles in the egg cytoplasm differ greatly to those in the previous stage; vesicles have almost disappeared, as has starch from the plastids (Fig. 4D, E), and mitochondria are increasingly prominent (Fig. 4B). In the VCC, vesicles and Golgi bodies increase above the fertilization pore (Fig. 4C, D), whilst mucilaginous material accumulates around this cell (Fig. 4C).

Nuclear evaginations.—During formation of the osmiophilic egg envelope, the original ellipsoidal nucleus of the egg changes into a cup-shape (Fig. 4A) and its surface produces numerous sac-like evaginations (Fig. 4E and F, Nev). These are usually between 0.5–0.7 μm in diameter and remain connected to the main body of the nucleus via narrow isthmuses (Fig. 4E, arrow). Sacs similar to the nuclear evaginations, but without connection to the nucleus in sections, occur the cytoplasm of the egg (Fig. 4F, arrows). The matrix of the evaginations resembles that of the main body of the nucleus (Fig. 4E, F).

The mature egg.—By the time the egg is mature the greatly shrunken VCC is surrounded by copious mucilaginous material, but still remains connected to the egg via the fertilization pore (Fig. 5A). Clusters of plastoglobuli have replaced starch in the VCC plastids (Fig. 5B). Parallel sheets of rough ER accumulate in the upper cytoplasm of the egg (Fig. 5 C, D) and evaginations remain numerous around the lower surface of the nucleus (Fig. 5C, F).

The transverse diameter of the fertilization pore almost reaches 2.8 μm (Fig. 5D). The membranes around the pore seem to be interwoven to form the thick border around the pore (Fig. 5D). Sometimes the vesicles containing osmiophilic material can be seen within and outside of the fertilization pore (Fig. 5D, Veo). The osmiophilic globules, as seen in previous stage, are also

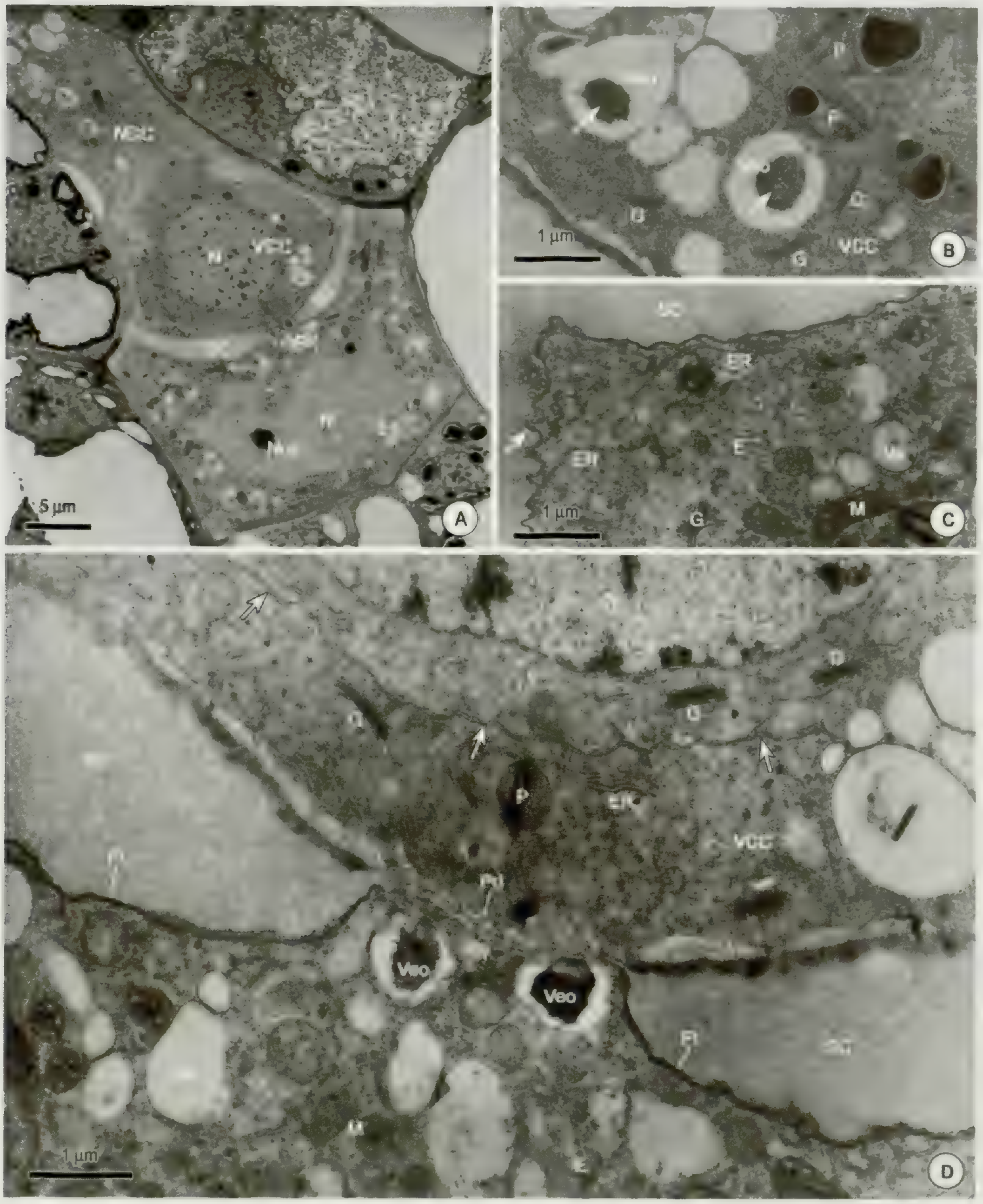
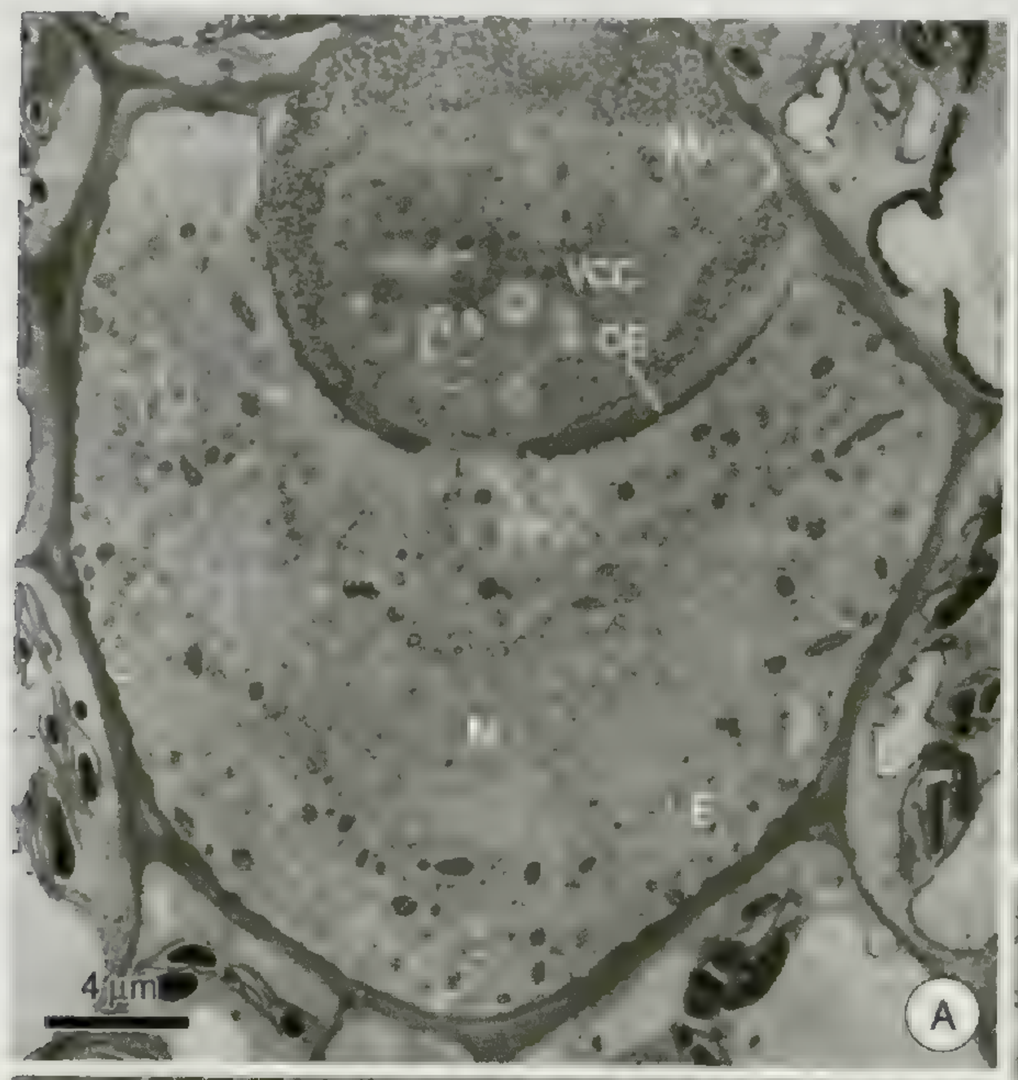
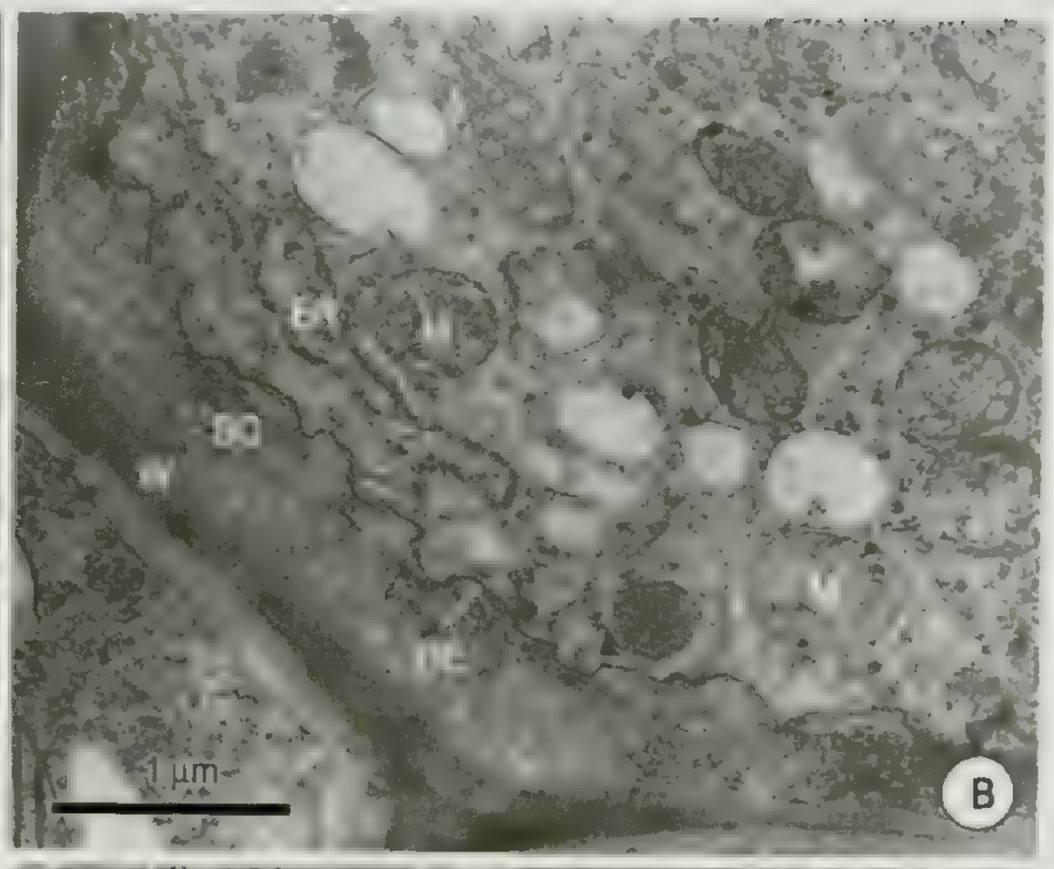
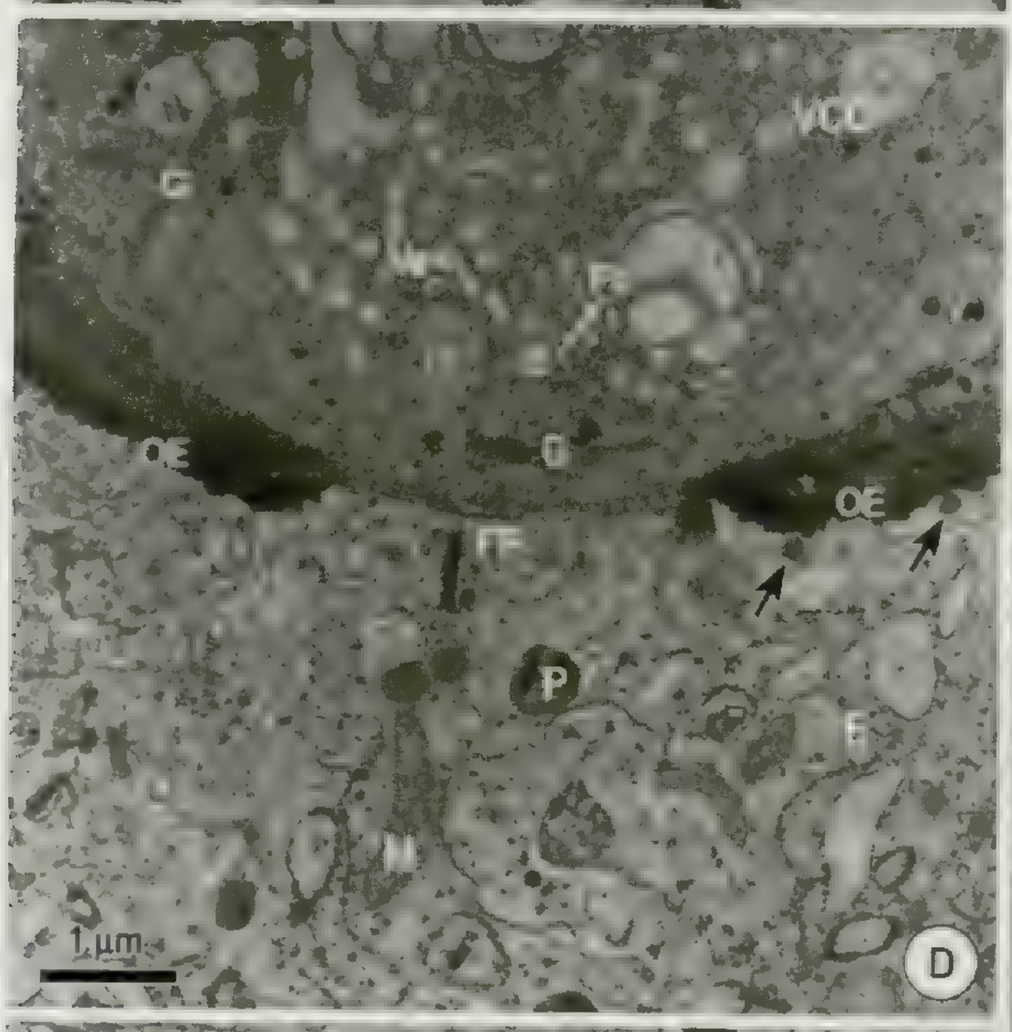
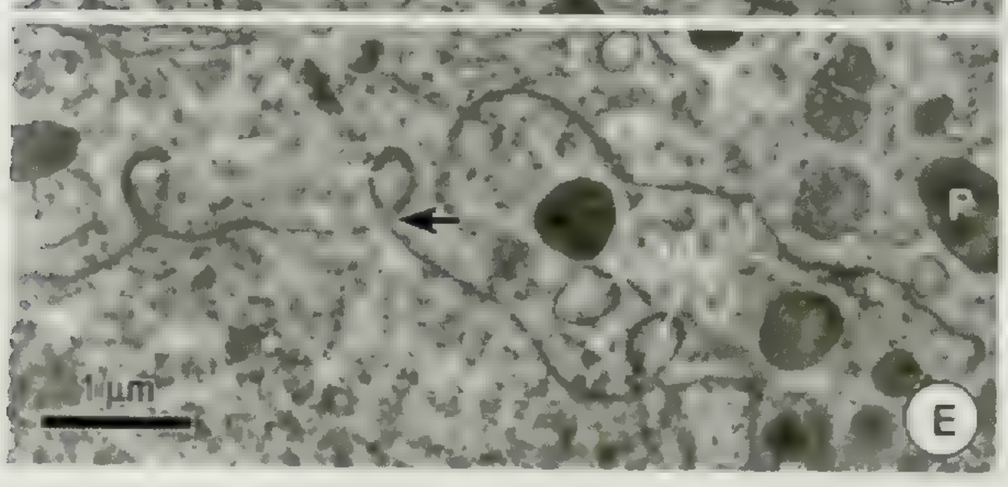


FIG. 3. Initiation of the fertilization pore. **A.** An archegonium containing a maturing egg (E), a VCC and a NCC. A pore region (PR) persistently connects the egg and VCC. The nucleus of the egg becomes slightly cup-shaped. **B.** Cytoplasm of the VCC showing the vesicles often containing the osmiophilic materials (Veo). **C.** Upper-side part of the egg, showing the dentate surface. **D.** Magnification of Fig. 3A. showing well- developed plasmodesmata (Pd) in the pore region. Vesicles containing the osmiophilic materials (Veo) below the pore region. A sheet-like mitochondrion (arrows) lies beneath the nucleus of the VCC; Golgi bodies (G), ERs and vesicles (Ve) are abundant in the cytoplasm of the VCC. M, mitochondria; N, Nucleus; Nus, nucleolus. P, plastid; Pl, plasmalemma; SC, separation cavity.









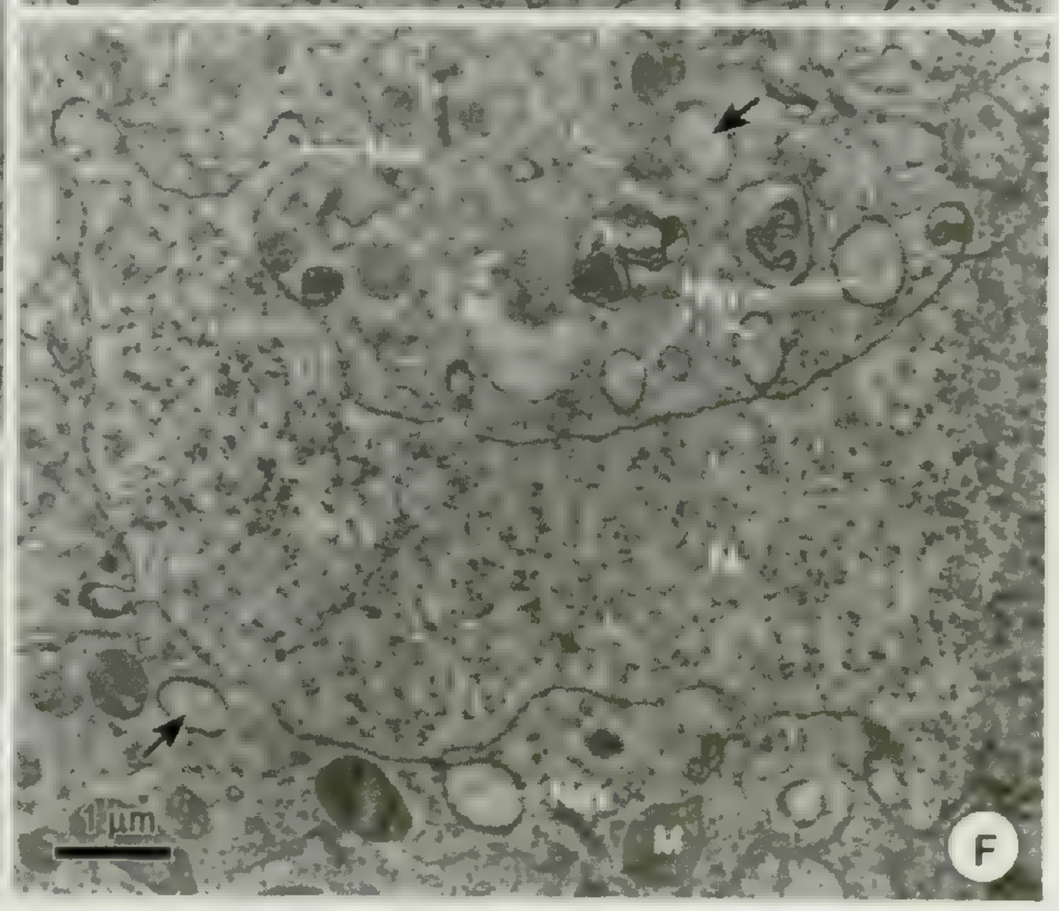


FIG. 4. The fertilization pore and nuclear evaginations. **A.** The egg cell is surrounded by a lay of osmiophilic envelope (OE). Fertilization pore (FP) on the upper surface of the egg. **B.** Lower part of the egg, showing the organelles. **C.** The osmiophilic egg envelope (OE) in the upper surface of the egg. Osmiophilic globules (arrows) are often seen next to the egg envelope. **D.** Section through the FP, the OE is absent in this region. Arrows indicate the osmiophilic glubles. **E.** Nuclear evaginations (Nev) usually connected to the main body of the nucleus by a narrow isthmus (arrow). **F.** Some of the evaginations are probably pinched off of the nucleus (arrows). E, Egg; M, mitochondria; N, Nucleus; Mu, mucilaginous materials; P, plastid; SC, separation cavity.

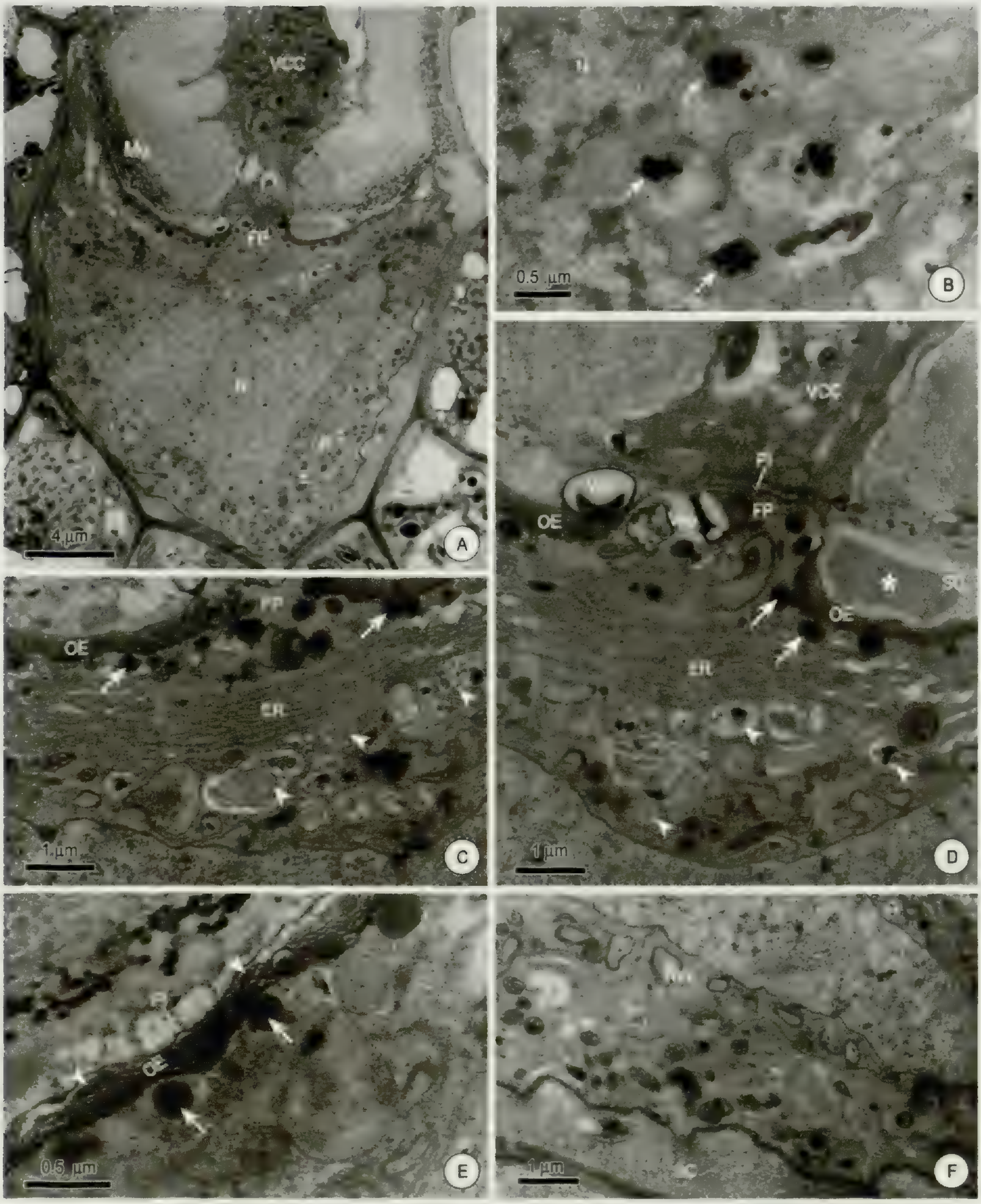


FIG. 5. Mature egg. **A.** A matured egg with a fertilization pore (FP). The VCC is degenerating. **B.** Part of VCC, showing degenerating plastids with clusters of plastoglobuli. **C, D.** Eggs showing pleomorphic vesicles with irregular membrane profiles and osmiophilic deposits at the isolated evagination (arrowheads). Arrows indicate the osmiophilic globules beside the osmiophilic egg envelope (OE). Vesicles containing osmiophilic materials (Veo) near and within the fertilization pore (FP). Electron-opaque materials (asterisk) in the separation cavity (SC). Sheets of endoplasmic reticula are seen in upper part of the egg cytoplasm. **E.** Magnification of the osmiophilic egg envelope showing its fibrillar structure. Osmiophilic globules are arrowed; arrowheads indicate osmiophilic materials within the osmiophilic egg envelope (OE). **F.** Numerous evaginations (Nev) along the lower surface of an egg nucleus. E, Egg; M, mitochondria; N, Nucleus; P, plastid; SC, separation cavity.

obvious beside the osmiophilic egg envelope (Fig. 5C–E, arrows). The egg envelope is composed of a stack of membranes (Fig. 5E). The osmiophilic globules resemble those beside the egg envelope filled in the spaces between membranes of the egg envelope (Fig. 5E, arrowheads). Electron opaque material (asterisk) can be seen in the SC (Fig. 5D).

Discussion

This study shows that the fertilization pore originally described in *Ceratopteris* (Cao *et al.*, 2009) is also formed during egg maturation in *Adiantum*. The transverse diameter of the fertilization pore in *Adiantum* reaches 2.8 μm, slightly larger than that of *Ceratopteris*, which diameter is about 2.5 μm (Cao *et al.*, 2009).

Although a separation cavity had been observed around the egg during oogenesis in a few ferns (Bell and Duckett, 1976; Bao *et al.*, 2003, 2005; Cao *et al.*, 2009), the details of its formation have not been described. The present study shows that the separation cavity first forms along the periphery of the upper surface of the egg. The centripetal expansion of the separation cavity gradually decreases the tissue connection between the egg and the VCC. Our observations confirm previous suggestions that the separation cavity is formed by the elimination of the vesicles from the egg cytoplasm (Bell and Mühlethaler, 1962b; Bell and Duckett, 1976; Bao *et al.*, 2003, 2005). As in *Ceratopteris* (Cao *et al.*, 2009), the absence of the osmiophilic egg envelope in *Adiantum* results in the formation of a fertilization pore.

The VCC almost certainly plays an important role in oogenesis. The persistent plasmodesmatal connections between the VCC and the egg will permit transfer of materials between the two including possible nutrient transfer to the egg (e.g., soluble carbohydrates from the starch in the VCC plastids) as the VCC breaks down. Alternatively the plastid starch may contribute to the mucilage secreted around the VCC from its maturational Golgi activity. In the later stage of the maturing egg, the nucleus of *A. flabellulatum* possesses distinctive evaginations, similar to those in other leptosporangiate ferns so far investigated, including *Pteridium aquilinum* (Bell and Mülethaler, 1962b; Bell and Duckett, 1976), *Dryopteris filix-mas* (L.) Schott (Cave and Bell, 1975), *Histiopteris incisa* (Bell, 1980), *Dryopteris crassirhizoma* (Bao *et al.*, 2005). However, the precise function of these structures, other than a general interpretation that they are a cytological manifestation of profound nucleocytoplasmic interactions preparing the eggs for future growth of the sporophyte (Bell and Duckett, 1976), remains unknown. Although the present study provides no evidence that the evaginations in *Adiantum* become detached as suggested for *Pteridium* (Bell, 1972), we speculate that the evaginations enter into the cytoplasm. The evaginations may eventually be digested in the cytoplasm of the egg as the egg matures, because some of them obviously become vacuolated (Fig. 5C, arrows).

Abundant membranous structures, probably ER, occur in the cytoplasm of the egg, which are also found in the mature eggs of the fern *Ceratopteris*

(Lopez- Smith and Renzaglia, 2008; Cao *et al.*, 2009). The membranous structures, occurring in the cytoplasm of the matured egg of *Adiantum*, may have an analogy to those in the egg of *Ceratopteris*, which is thought to prevent polyspermy (Cao *et al.*, 2009). The occurrence of the membranous structures probably indicates that the egg has become fertilizable.

ACKNOWLEDGMENTS

The authors thank Ms. Hui-Qi Zhang for help with transmission electronic microscopy. This research was supported by National Natural Science Foundation of China (30970267, 30670128), Leading Academic Discipline Project of Shanghai Municipal Education Commission (J50401) and Project of Shanghai Municipal Education Commission (08YZ67).

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American Fern Journal 100(2):103–109 (2010)

Gametophyte of the Andean Fern *Polystichum pycnolepis* (Kunze ex Klotzsch) T. Moore (Dryopteridaceae)

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Abstract.—The gametophytic generation of *Polystichum pycnolepis* (Dryopteridaceae), including spore germination, morphological development of the gametophytes, major vegetative features and sexual expression, was studied. Spore germination was of the *Vittaria* model and the developmental pattern was of the *Aspidium* model. Adult gametophytes were cordate and hairy, with unicellular hairs located in the margins and the surface of the prothalli. In addition, hairs of *P. pycnolepis* were papillate, secretory and the marginal ones septate. Gametangia were of the normal type described for leptosporangiate ferns. The gametophytes produced at first instance a high proportion of female gametophytes. *Polystichum pycnolepis* formed few bisexual gametophytes with scarce antheridia. No male gametophytes for this species were detected. Thus, the species seemed to be promoting intragametophytic selfing and, to a lesser extend, intergametophytic unions between bisexuals and females.

Key Words.—gametophyte, development, reproduction, Andes, Peru, *Polystichum pycnolepis*

Polystichum Roth. (Dryopteridaceae) is a large genus, with more than 250 species, worldwide in distribution (Tryon and Tryon, 1982). It is rare or absent in lowland tropical areas and best represented in the mountains of tropical and warm-temperate climates (Kramer, 1990) with a great diversity in eastern Asia (Lu *et al.*, 2007). Around 70 species are present in the neotropics. *Polystichum pycnolepis* (Kunze ex Klotzsch) T. Moore is a terrestrial montane plant that occurs in Colombia, Venezuela, Ecuador, Peru and Bolivia. Despite the diversity of species of *Polystichum*, not much research has been done on its gametophytic phase (but see Paterson, 1942; Nayar *et al.*, 1968; Nayar and Kaur, 1969; Chandra and Nayar, 1970; Sato and Saki, 1980; Greer and McCarthy, 1997; Muccifora and Gori, 1997; Pangua *et al.*, 2003; Nazario and Arreguín, 2007), and much less with the neotropical species.

The aim of this work was to study spore germination, the prothallial development, the morphology of the adult gametophyte and the sexual behavior of *P. pycnolepis*.

Material and Methods

Spores for cultures were taken from sporophytes collected in Peru, Cuzco Department, Urubamba Province. The following are the locations of the

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collections: de Ollantaytambo hacia el Puerto de Málaga, 13°10′36.9″S 72°17′19.4″W, 3.440 m, ribera umbrosa de un arroyo, *Gabriel y Galán* s/n, 29/04/2008. The material was identified following Tryon and Stolze (1991). Vouchers are deposited in MA.

Each spore sample for cultures were taken from separate sporophytes kept dry at room temperature for around 8 months. Gametophytes were grown under fluorescent light on a 12-h light, 12-h dark cycle at 20 ± 2°C, in 6 cm plastic Petri dishes. Multispore cultures on mineral agar medium (Dyer, 1979) were established by sacking fertile pinnae on a weigh paper, and placing the obtained spores in the Petri dishes. The sowing was replicated twice for each sample. Percentage of germination was recorded on every third day for a random sample of 50 spores from each of the two plates, until the maximum germination was reached. Spore sizes given are the mean of 30 measurements. To study the stages of gametophyte development, random samples were taken weekly, from the beginning of spore germination until sexual maturity. Gametophytes were stained with chloral hydrate acetocarmine (Edwards and Miller, 1972), mounted in water and observed under a light microscope. Some in vivo observations were also made.

Results

The spores of *P. pycnolepis* were monolete, ellipsoidal, 48.0 × 34.5 µm in equatorial view, with an irregular cristate perispore. The first sign of germination was detected after 7 days from sowing, when some spores (ca. 5%) showed a rhizoid; a few days later, the first prothallial cell appeared, growing perpendicular to the rhizoid. Germination percentage was relatively low, as the maximum percentage achieved was of ca. 30%, around 21 days after sowing. Figure 1 shows the progress of the germination percentages over the days considered.

In *P. pycnolepis*, the prothallial cell emerged from the spore and divided to form a short filament of 3–5 cells with a length of 90–120 µm (Fig. 2A). At this stage, ca. 7 days after germination, the terminal cell of the filament developed an apical hair that limits its growth. The apical cell divided longitudinally, so the hair was finally located in one of the daughter cells, which became somewhat quiescent. Further divisions of the apical daughter cell without hair and by the subapical cells composed the planar plate (Fig. 2B). For this lop sided divisions, the bidimensional plate was somewhat asymmetric in the apical region, but soon, ca. 30 days after germination, this asymmetric condition was lost and a pre-cordate symmetric shape was reached (Fig. 2C). Hairs were continuously formed while the meristem developed a clear notch and the wings (Fig. 2D). Mature pre-sexual gametophytes arose ca. 60 days after germination, and were of the cordate hairy type, of about 4 mm in width, with ample, symmetric, well-developed wings (Fig. 2E).

The gametophytes of *P. pycnolepis* began to form marginal unicellular capitate hairs (Figures 2C–E, 3A), of about 30–40 µm in length, after 20 days of germination. Superficial hairs developed after ca. 50 days of germination,

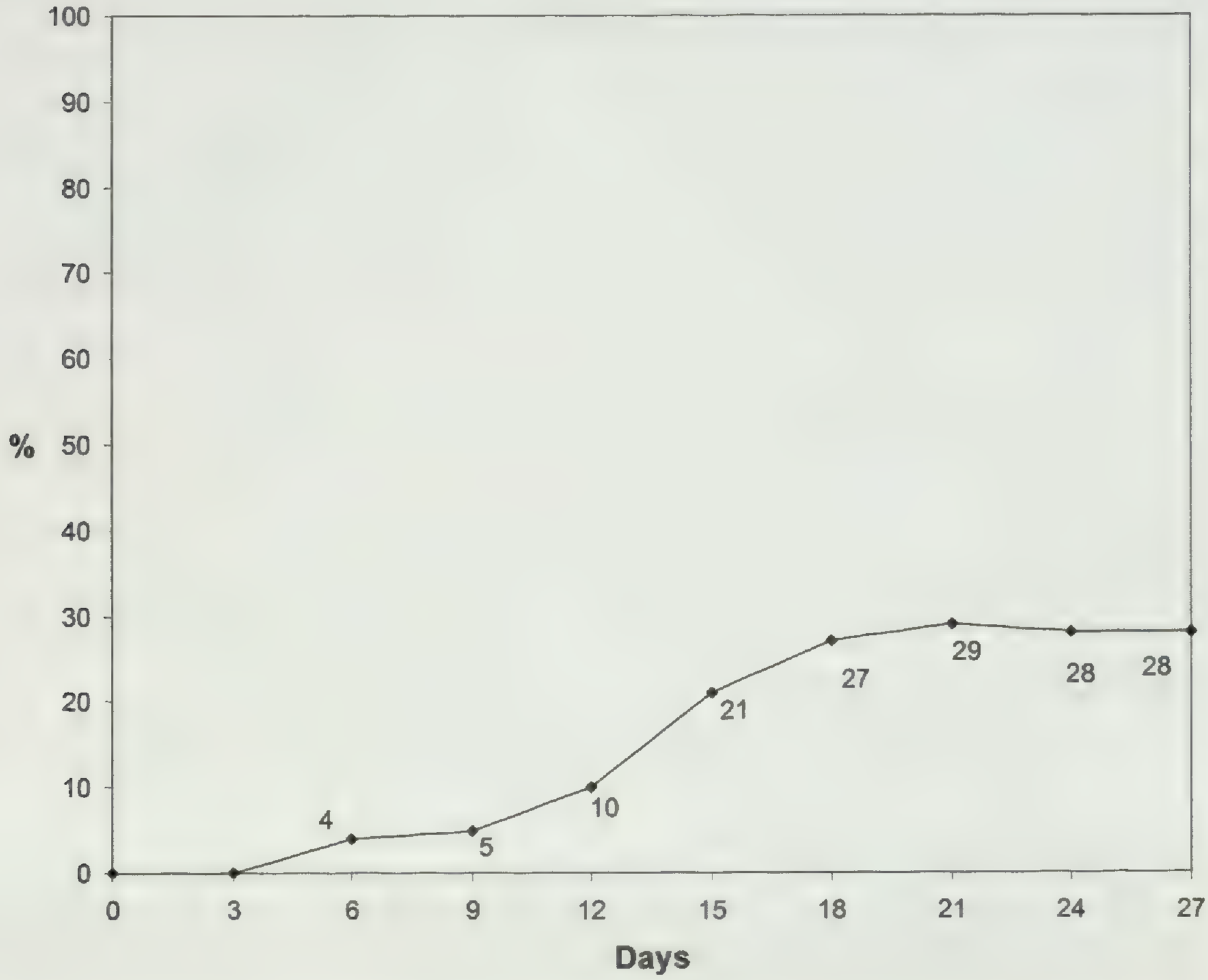

FIG. 1. Changes in the germination percentages of *Polystichum pycnolepis*, over 27 days.

achieving its maximum density during the gametangia formation around the archegonia in the ventral surface. These superficial hairs were also unicellular and capitate, but longer than the marginal ones, of about 50–60 μm. When the prothalli reached the adult stage, most of the marginal hairs became secretors, accumulating an apical coat of extracellular secretion (Fig. 3A). In this adult stage, the apical region of both the marginal and the superficial hairs changed to become papillate. At the same time, the base of the marginal hairs formed a somewhat undulate septum (Fig. 3B).

About 60–70 days after germination, gametangia were observed in some prothalli of *P. pycnolepis*. Antheridia formed in small numbers and on some prothalli just one antheridium was observed (Fig. 3C), while archegonia were abundant (Fig. 3D). From the beginning of the sexual stage until the end of the observation period (ca. 250 days from germination), female and rarely bisexual prothalli were formed, and no male gametophytes were observed.

DISCUSSION

The size and ornamentation of the spores of *P. pycnolepis* fall within the ranges of variation and types previously reported for the genus (Tryon and Lugardon, 1990). The spore germination is of the *Vittaria* type (Nayar and

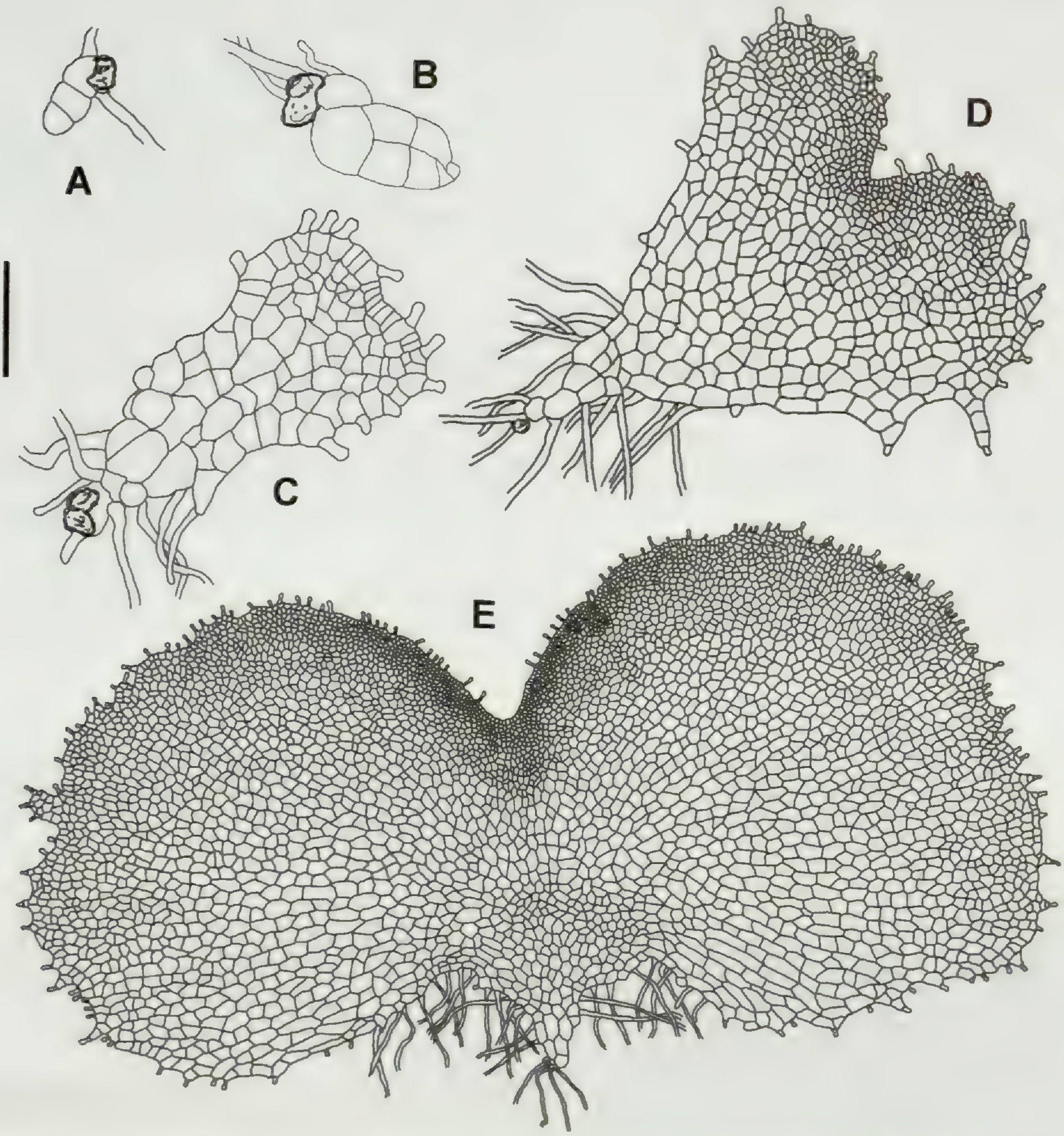

FIG. 2. Gametophyte development of *Polystichum pycnolepis*. A: filament stage, 7 days. B: initial planar stage, 15 days. C: planar non cordate gametophyte, prior to cordate stage, 30 days. D: cordate immature stage, 40 days. E: adult prothallus, 60 days. Days are measured from the germination. Bar= 90 μm in A, B; 160 μm in C; 350 μm in D; 500 μm in E.

Kaur, 1968), which has been reported on other *Polystichum* species (Chandra and Nayar, 1970; Nazario and Arreguín, 2007).

The prothallial development found in *P. pycnolepis* is of the *Aspidium* type (Nayar and Kaur, 1969), in which the germination filament ends in a hair. This kind of development is usual in the Dryopteridaceae (Nayar and Kaur, 1969), and it has been reported in many of the principal genera (Mendoza *et al.*, 1999; 2002), including *Polystichum* (Chandra and Nayar, 1970; Atkinson, 1973). Over the general *Aspidium* type described by Nayar and Kaur (1969), several

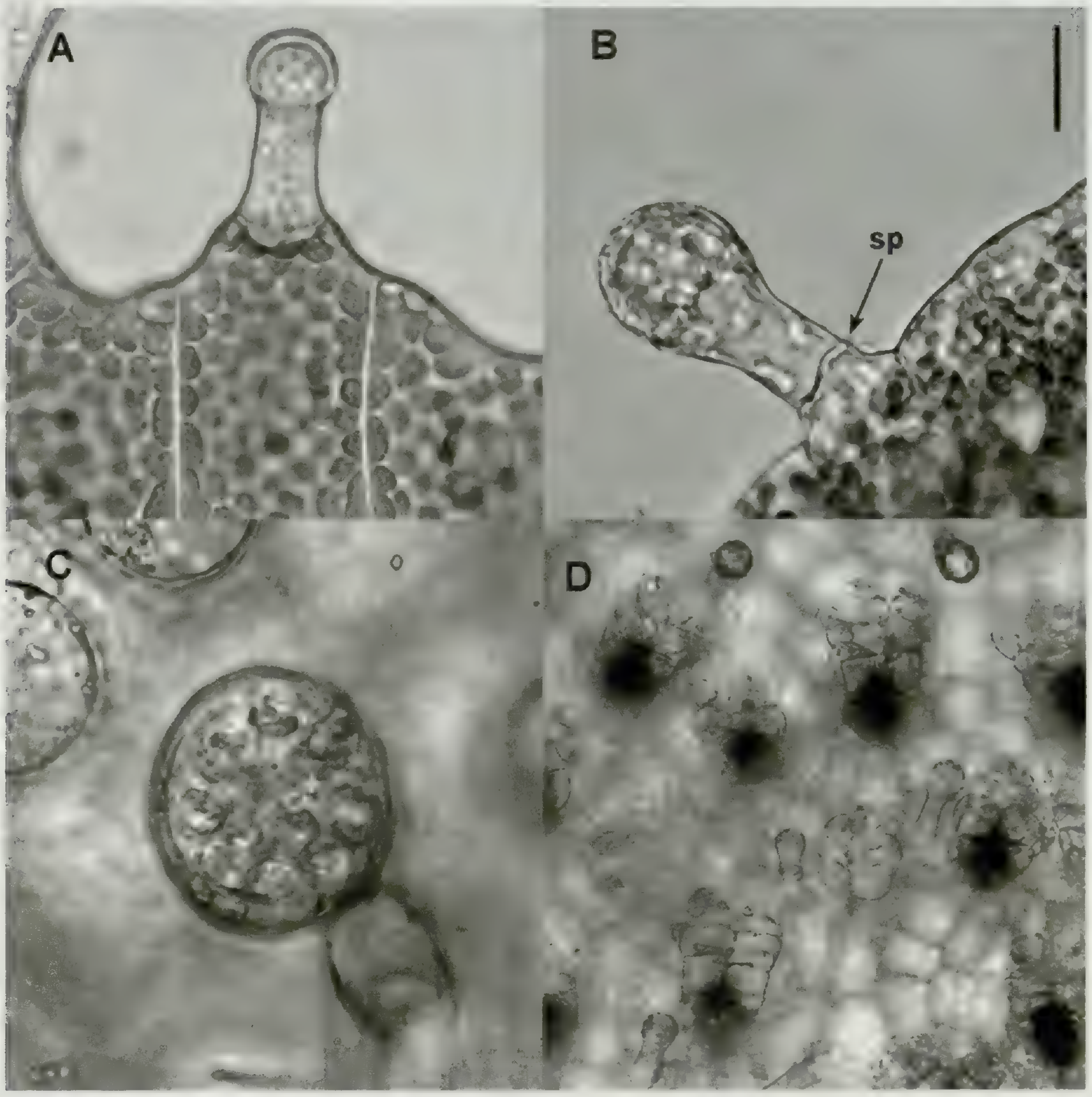

FIG. 3. Vegetative and reproductive features of *Polystichum pycnolepis*. A: marginal unicellular hair on an adult pre-sexual prothallus, 60 days. B: marginal septate papillate hair, in an old sexual prothallus, 180 days; sp=septum. C: antheridium, 180 days. D: archegonia, 180 days. Days are measured from the germination. Bar = 20 μm in A; 15 μm in B, C; 50 μm in D.

variations have been reported. *Polystichum pycnolepis* deviated from the general model in the formation of a longitudinal division of the apical cell, after the formation of the apical hair, so this is located in one of the daughter cells. This fact has been also found in other *Polystichum* species, for example in *P. biaristatum* (Blume) T. Moore., while others, as *P. auriculatum* (L.) C. Presl, do not divide the apical cell (Nayar and Kaur, 1969).

Unicellular papillate secretory hairs as those of *P. pycnolepis* have been found in several Dryopteridaceae taxa, as in other *Polystichum* species (Chandra and Nayar, 1970; Nayar and Kaur, 1971; Nazario and Arreguín, 2007), *Dryopteris* (Pérez García *et al.*, 1999), *Olfersia* (Mendoza *et al.*, 2002)

and *Arachniodes* (Chandra and Nayar, 1970; Mendoza *et al.*, 1999). In these species, hairs are non septate or this character is unknown, while basally septate hairs, as those found in *P. pycnolepis*, have been reported from genus of related families, as *Cyclopeltis* (Stokey, 1960). In some other *Polystichum* species marginal non secretory swollen hairs and papillate secretory hairs have also been found mixed (Nayar and Kaur, 1971). In *P. pycnolepis* the superficial hairs are longer than the marginal ones, a pattern that seem to be normal (Nayar and Kaur, 1971).

The sex organs in *P. pycnolepis* are typical for leptosporangiate ferns (Nayar and Kaur, 1971). The rare formation of antheridia in *P. pycnolepis* can be interpreted in various ways. Unfavorable conditions tend to increase the maleness of prothalli whereas extremely favorable conditions, as artificial cultures, could cause in the formation of strictly archegoniate prothalli (Nayar and Kaur, 1971). In a more detailed study, some *Polystichum* species, as *P. ordinatum* (Kunze) Liebm., have been reported to form the male gametangia at an advanced age, around 366 days after germination (Nazario and Arreguín, 2007). But in this case, also archegonia were formed later (218 days) than in *P. pycnolepis* (70 days). The observation in *P. pycnolepis* of antheridia coexisting with archegonia in an early sexual stage of development implies a very different strategy than the observed in *P. ordinatum*; in the latter, intergametophytic crossing seems to be promoted, as only unisexual prothalli have been detected. Other authors have mentioned the influence of light in the development of gametangia, in the sense that an intense light source could inhibit the formation of antheridia (Paterson, 1942; Raghavan, 1989). In some of these experiments, apogamous *Polystichum* species were involved, as *P. tsus-simense* (Hook.) J. Sm. Apogamy is known to be present in the genus, but that is not the case of *P. pycnolepis*; again, the reproductive strategy in *P. pycnolepis* is very different than that of *P. tsus-simense*. Some other studies (Pangua *et al.*, 2003) with the European species *P. aculeatum* (L.) Roth ex Mert. and *P. lonchitis* (L.) Roth, reported the presence of only bisexual prothalli, while *P. setiferum* (Forssk.) Moore ex Woyn. had both female and bisexual prothalli. This latter reproductive strategy, despite the limited presence of antheridia, is likely to be happening in *P. pycnolepis*, which would be favoring both intergametophytic and intragametophytic crossings. Nevertheless, both *P. aculeatum* and *P. setiferum* had an antheridiogen system, which is not operating in *P. pycnolepis*.

Acknowledgments

Authors thank the financial support for the field trip to the Spanish Red Universitaria de Investigación sobre Cooperación para el Desarrollo (project G07/10).

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American Fern Journal 100(2):110–125 (2010)

Ecology and Syntaxonomy of *Gymnocarpium dryopteris* L. in the Netherlands

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ABSTRACT.—Ecology, syntaxonomy and population size of *Gymnocarpium dryopteris* have been studied, especially during the period 1979–1991 by collecting data on 419 colonies in the Kuinderbos (a planted woodland in the Noordoostpolder, a polder reclaimed from the former Zuiderzee in 1942) and 74 colonies elsewhere in the Netherlands. In this paper the Kuinderbos population is compared with other Dutch populations (pleistocene area) in order to provide more insights in the ecology of the species and circumstances facilitating high density. Populations in Germany were studied as reference. In the period 1979–1991 the species was recorded in the Netherlands with at least 670 colonies, of which 89% are in the Kuinderbos. *Gymnocarpium dryopteris* prefers shadowed ditches and drainage trenches, but can grow at various other habitats as well. Especially at the western and southern part of the Netherlands, the species grows on canal walls. Sites with *Gymnocarpium dryopteris* belong to different communities. In the Kuinderbos stands of *Picea sitchensis* are preferred, while elsewhere in the Netherlands the species prefers the *Quercion roboris-petreae* (*Fago-Quercetum*) or *Pseudotsuga mensiezii*-stands. *Gymnocarpium dryopteris* is accompanied by 14 fern species, at the Kuinderbos by 12 fern species, with *Athyrium filix-femina* as characteristic species, often indicating potential sites. Rhizomes are shallow creeping, at average 2.6 cm below the surface (0.5–8 cm). It grows in the ecto-organic layer or just below this layer. At trench sides it often grows in the mineral layer. Most colonies are within the influence of the watertable. The Dutch colonies are small and colonies with more than 1000 fronds are rare. This probably indicates that most colonies are less than 20 years old. Colonies with more than 100 fronds are often fertile, with less than 20% of the fronds bearing sori. At the wall habitat small-sized individuals may be fertile as well. The high density in the Kuinderbos can be attributed to an optimal water supply from a peat subsoil, the composition of the ecto-organic layer consisting of *Picea* needles and an optimal light climate. The high density in *Picea sitchensis* within the Kuinderbos is unprecedented in Europe and makes these stands resemble the natural habitat of *Picea sitchensis* at the west coast of N. America.

KEY WORDS.—Gymnocarpium, syntaxonomy, ecology

Though *Gymnocarpium dryopteris* L. is one of the rarest fern species in the Netherlands (Mennema *et al.*, 1985), it is not endangered (van der Meijden *et al.*, 2000). Since 1979, many new sites of this species have been discovered, the most important being in the Kuinderbos, one of the woodlands planted in the former Zuiderzee (Noordoostpolder). The large number of sites where the density of the species is locally high has provided an opportunity to describe the ecology of the species in this woodland (Bremer, 1980; Bremer, 2007). In the Kuinderbos, *G. dryopteris* is closely associated with *Picea sitchensis* (Bong.) Carrière, whereas elsewhere in the Netherlands the species has been recorded in woodlands, on stream banks and sometimes from unlikely habitats, such as canal walls (Weevers *et al.*, 1948; van Ooststroom, 1973).

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This raised the question: what are the optimal ecological conditions for *G. dryopteris* in the Netherlands to develop and form large patches?

First, it was important to do a thorough ecological study of the large population in the Kuinderbos. Subsequently a more detailed study of colonies elsewhere in the Netherlands was initiated, to ascertain the conditions under which the species is able to establish but is seldom able to form a population of appreciable size, and also to look at the types of vegetation in which *G. dryopteris* occurs. It is the composition of the vegetation and the abundance of its constituent species that provide information about the abiotic conditions and management (e. g., Grootjans, 1985; Schaminée *et al.*, 1995), in so far that constraints to dispersion do not strongly influence the composition of the vegetation, and provided the communities are saturated (no impact of seed or spore limitation). When comparing populations, aspects of performance were also considered, measured from the size and fertility of the colonies. It is the small and young colonies that provide information about the potential for establishment: the microhabitat in which they have established is often still discernable in the field. The colonies that have been able to survive in certain places for a long time yield different information. The environment in which prothallia can establish successfully may differ greatly from the environment of the mature colonies, as is the case for *Pteridium aquilinum* (L.) Kuhn (Page, 1982).

Material and Methods

A questionnaire on the characteristics of the locality and site was compiled and sent out to botanists and vegetation scientists who had sent in observations to the National Herbarium (Leiden University). In total, 26 respondents completed the questionnaire or supplied other information. In addition, documentation at the National Herbarium was consulted – including the descriptions in the herbarium collection. I visited 60% of the sites in the Netherlands where the species has been found and described them. Most of the data on the Kuinderbos were collected in 1979 (Bremer, 1980). Data on the other sites elsewhere in the Netherlands were collected from 1979 to 1990.

In the field the composition of the tree layer was noted. The soil composition was determined by taking multiple samples of the top 30 cm with a soil auger and describing the soil composition (e.g., Wiggers *et al.*, 1962). The pH was determined at 28 locations from soil collected from the root zone (ecto-organic layer plus soil from the underlying 5 cm layer). This entailed collecting 15 g of soil and mixing it with 20 ml of distilled water (pH H_20) in the laboratory. The next day the mixture was shaken and then analyzed. The pH-KCl was measured after adding 2.5 ml 1 N KCl. The thickness and composition of the ecto-organic layer were determined by carefully scraping off this layer several times in each colony (Klinka *et al.*, 1981). In the Kuinderbos, the water table was determined from the water levels in piezometers (filter at 80–100 cm). Elsewhere, the mean highest water table (MHWT) was estimated from the water table in nearby watercourses or using soil features (rust phenomena).

The associated fern species were those within a radius of 3 m from the edge of a patch. If there were microtopographic differences in the site (as was

particularly the case in drainage trenches) slope was measured with a clinometer. Aspect was determined from the topographical map. In all colonies the number of fronds colony^{-1} and the number of fertile fronds were counted. The data supplied by respondents were not always complete. This accounts for the differences between the numbers of colonies for which data on the aforementioned parameters are available.

Vegetation relevés were made in the habitats the species had been found (n = 37 sites). In woodlands these relevés were 10 × 15 m in size, in other habitats smaller. Data were analysed with TWINSPAN (Hill, 1979). Twinspan analyzes sets of relevés by calculating the similarity between all possible combinations. Relevés with high level of similarity are grouped in the same cluster and later on syntaxonomically interpreted.

Data on vegetation and other aspects were also collected from colonies in four mountain areas in Germany (Harz, Weserbergland, Teutenburgerwoud, Allgäu). Chi-square tests were used to compare the actual distributions of *G. dryopteris* over various habitats versus those that would be predicted. The scientific names in this paper are based on van der Meijden (2005).

Results

Ecology.—Gymnocarpium dryopteris can be found in a large number of different habitats in the Netherlands (Table 1). There are significant differences in the distribution of the sites over the various habitats in the Netherlands prior to and since 1979 ($\chi^2 = 52.8$, $p < 0.001$, d.f. = 5). Prior to 1979, the species was found in a larger number of different habitats. In the period since 1979, the habitat preference in the Kuinderbos has been very different to that elsewhere in the Netherlands ($\chi^2 = 305.0$, $p < 0.001$, d.f. = 8); it is strongly related to the great preference in the Kuinderbos for trenched coniferous woodland. *Gymocarpium dryopteris* occurs mostly in locations with some differences in microtopograhic position: this applies to 89% of the clones in the Kuinderbos and 75% of the clones elsewhere in the Netherlands. The differences in microtopographic position are often associated with sides of trenches, but this category also includes walls. In the past, the species has also been recorded on pollarded willows, in wells and on wooded banks, but there have been no recent sightings in these habitats. Only since 1984 have individual specimens been recorded growing between the basalt blocks on former sea dikes (Lauwersmeer and IJsselmeer dikes: van der Ploeg, 1984).

In the Netherlands, *G. dryopteris* occurs most in stands of *Pseudotsuga menziesii* (Mirb.) Franco and *Quercus robur* L. Eighty seven percent of sites in the west and south of the Netherlands have something to do with walls (n = 15). In the Kuinderbos, 79% of the clones were found under *Picea sitchensis* (Table 2). The distribution over Kuinderbos sites with different tree species is significantly different from that elsewhere in the Netherlands (period 1979–1981) ($\chi^2 = 289.1$, $p < 0.001$, d.f. = 5). For the entire country and the period 1979–1991, two clones (3.1%) occurred under *Fagus sylvatica* L. In the Kuinderbos there was also a different preference in relation to the distribution

TABLE 1. Habitat preference of *Gymnocarpium dryopteris* in the Netherlands. The table shows the number of colonies per habitat. A distinction is made between populations that disappeared prior to 1979 and have not been recorded since, populations present in the period 1979–1991 (Pleistocene area in particular) and populations in the Kuinderbos (benchmark population: the entire population in 1979) (Values ≤ 3 were clustered in the chi-square analysis in order to fulfil the requirements of this test).

	Pleistocene area				Kuinderbos	
	Number of colonies before 1979	%	Number of colonies 1979–1991	%	Number of colonies in 1979	%
Soil under conifer stands			6	9.2	57	13.6
Soil under broadleaved stands			9	13.8	1	0.2
Trench in coniferous woodland			9	13.8	347	82.8
Trench in deciduous woodland			1	1.5	14	3.3
Not shaded ditch/drainage trench			10	15.4	-	
subtotal woodlands/trench	24	35.8	35	53.7		
Stream	8	11.9	8	12.3	-	
Canal wall/quay wall	16	23.9	8	12.3	-	
Brick wall (dry)	8	11.9	2	3.1	-	
Basalt dike	-		9	13.8	-	
Roadside verge	-		3	4.6	-	
Wooded bank	3	4.5	-		-	
Drainage canal	2	3.0	-		-	
Pollarded willow	2	3.0	-		-	
Well	2	3.0	-		-	
Hollow way	1	1.5	-		-	
Edge of steep drop	1	1.5	-		-	
Total	*67*	*100*	*65*	*100*	*419*	*100*

of the top 10 cm of soil compared with the rest of the Netherlands ($\chi^2 = 105.2$, $p < 0.001$, d.f. = 9) (Table 3). In the Netherlands, *G. dryopteris* occurs most often on moderately fine, coarse and loamy sandy soils, whereas in the Kuinderbos the soil composition is humic to peaty fine sand. Outside the Kuinderbos the soil is lime-poor with a measured pH-KCl of 3.9 (± 1.0, n = 12). In the Kuinderbos the fine sand is always calcareous, whereas peat and clay are lime-poor. In 52 of the 141 sites studied where clones were found, limy material occurred in the top 10 cm (Bremer 1980) and the mean pH-KCl was 6.6 (± 0.9, n = 16).

The average maximum thickness of the ecto-organic layer within patches is 4.7 cm outside the Kuinderbos but 1.5 cm in the Kuinderbos (Table 4). The difference is not statistically significant ($\chi^2 = 9.2$, $p = 0.06$, d.f. = 4). The thin average ecto-organic layer in the Kuinderbos is associated with the marked preference for trench banks. On these banks the ecto-organic layer is either less developed or is absent due to land slip, while litter decomposition is faster because the pH is high. Here, the rhizomes are at a shallow depth in a basic soil, whereas at the top of the trench, fronds of the same clone are usually growing in the ecto-organic layer, where the pH is much lower (Table 5). Elsewhere in the Netherlands, the pH at the top of trenches and on the trench sides is low.

TABLE 2. Habitat preference in relation to dominant tree species in the tree layer. A distinction is made between populations that disappeared prior to 1979 and have not been recorded since, populations present in the period 1979–1991(Pleistocene area in particular) and populations in the Kuinderbos (benchmark population: 1979). * indicates no information on tree species (Values ≤ 3 were clustered in the chi-square analysis in order to fulfil the requirements of this test).

	Pleistocene area				Kuinderbos	
	Number of colonies before 1979	%	Number of colonies in 1979–1991	%	Number of colonies	%
Pseudotsuga menziesii	2	3.0	16	25.1	-	
Pinus spp.	5	7.5	2	3.1	-	
Picea abies	1	1.5	2	3.1	5	1.2
Quercus robur	1	1.5	10	15.6	53	12.6
Picea sitchensis	-		2	3.1	325	77.5
Fagus sylvatica	-		2	3.1	5	1.2
Larix sp.	-		2	3.1	2	0.5
Betula spp.	-		5	7.8	-	
Other tree species	22*	32.8	5	7.8	29	6.9
Other habitats	36	53.7	18	28.2	-	
Total	*67*	*100*	*64*	*100*	*419*	*100*

Shallow rooting also occurs in places where clones are growing in roadside verges and where there is no accumulation of litter. Here, the rhizomes grow in the mineral soil. Where colonies are growing on level ground under *Picea spp.* or *Pseudotsuga mensiezii*, the rhizomes may be growing in the mineral layer or the fermentation layer (litter layer), depending on the thickness of both these layers. At one site clones are growing in a trench with 2–10 cm litter overlying a fermentation layer that is up to 8 cm thick. Here, the rhizomes are mostly growing in the litter layer. The rooting depth varied from 0.5 to 8 cm.

The mean rooting depth based on measurements in three areas in the Netherlands is 3.3 cm (n = 82, Table 5a–c), in the Kuinderbos 1.8 cm (four

TABLE 3. Composition of the top 10 cm of soil under clones of *Gymnocarpium dryopteris* in the Netherlands (particular Pleistocene area, period 1979–1991) and in the Kuinderbos (1979).

	Pleistocene area		Kuinderbos	
Soil composition	Number of colonies	%	Number of colonies	%
Humic, moderately fine sand	16	27.2	-	
Moderately coarse sand	14	23.7	16	11.3
Sand on loam or loamy sand	10	16.9	-	
Stony substrate	10	16.9	-	
Between basalt blocks	8	13.6	-	
Loam	1	1.7	-	
Humic, very fine sand	-		59	41.9
Peaty, very fine sand	-		27	19.1
Peat	-		30	21.3
Clay	-		9	6.4
Total	*59*	*100*	*141*	*100*

TABLE 4. Thickness of the ecto-organic layer in sites of *Gymnocarpium dryopteris* in the Netherlands (Pleistocene area in particular) and in the Kuinderbos. The highest value is given for each clone.

	Pleistocene area		Kuinderbos	
Thickness (cm)	Number of colonies	%	Number of colonies	%
0.0–2.0	6	31.6	67	68.0
2.1–4.0	3	15.8	18	18.0
4.1–6.0	4	21.0	11	11.0
6.1–8.0	1	5.3	3	3.0
8.1–10.0	5	26.3	0	0.0
Total	*19*	*100*	*99*	*100*
mean	*4.7*		*1.5*	

populations together, 2.6 cm). Rooting depth also varies greatly in natural sites outside the Netherlands (Table 5e–f). On walls, the rhizomes of *G. dryopteris* grow shallowly, between the bricks. In the Kuinderbos, most clones are growing within the influence of the groundwater. This is particularly true for the clones growing lowest down the sides of trenches. Sometimes these clones endure brief soaking or even submergence. Elsewhere in the Netherlands, clones (or their parts) also generally occur within the influence of groundwater, but they can live outside this influence too (Table 6; $\chi^2 = 23.3$, $p < 0.001$, d.f. = 4). Many clones (55.3% outside the Kuinderbos, 88.5% in the

TABLE 5. The depth of rhizomes at various sites in the Netherlands and elsewhere. The measurements are based on 1–4 different colonies per area. The depth for each clone has been determined for places where the stipes are attached to the rhizomes. n = number of observations. **a.** Delden, shady roadside drainage trench. **b.** Lonnekerberg, shady roadside drainage trench, **c.** 't Velde estate, shady trench, **d.** Kuinderbos, clones on forest soil and trench sides under *Picea sitchensis*, **e.** Harz, Bremketal, in *Luzulo-Fagetum*, on mountainside, 51 40′N 10 20′E, **f.** Allgäu (southern Germany), Wiederhofen, in *Abieti-Fagetum* on mountainside 47 26′ N 10 25′E. Total = total number of determinations.

	Netherlands								Germany			
	Pleistocene area						Kuinderbos					
Depth	a		b		c		d		e		f	
(cm)	n	%	n	%	n	%	n	%	n	%	n	%
1	10	71.4	17	100	-		32	42.7	-		24	48.0
2	-		-		6	11.8	16	21.3	4	6.2	18	36.0
3	4	28.6	-		1	2.0	9	12.0	8	12.3	5	10.0
4	-		-		7	13.7	12	16.0	21	32.3	3	6.0
5	-		-		8	15.7	2	2.7	20	30.8	-	
6	-		-		19	37.2	4	5.3	3	4.6	-	
7	-		-		9	17.6	-		5	7.7	-	
8	-		-		1	2.0	-		3	4.6	-	
9	-		-		-		-		1	1.5	-	
Total	*14*	*100*	*17*	*100*	*51*	*100*	*75*	*100*	*65*	*100*	*50*	*100*
mean	*1.3*		*0.5*		*4.8*		*1.8*		*4.3*		*1.5*	

TABLE 6. Mean highest water table (MHWT) in metres for clones of *Gymnocarpium dryopteris* in the Netherlands (in particular in Pleistocene area) and in the Kuinderbos.

	Pleistocene area		Kuinderbos	
MHWT	Number of colonies	%	Number of colonies	%
≤ 0.4	15	38.4	23	46.0
0.41–0.8	4	10.3	27	54.0
0.81–1.2	1	2.6	-	
> 1.2	9	23.1	-	
on walls	10	25.6	-	
Total	*39*	*100*	*50*	*100*

Kuinderbos, both for the period 1979–1991) grow on unstable, steep, trench sides. The large difference between the Kuinderbos and the rest of the Netherlands in the slope of the sites ($\chi^2 = 39.5$, $p < 0.001$, d.f. = 8) is because outside the Kuinderbos the sites include walls and quays (with a slope of 80°–90°), whereas in the Kuinderbos many clones are growing on the level woodland floor (Table 7). Within the Dutch population, the species is found significantly more often on northerly aspects than on southerly aspects ($\chi^2 = 8.2$, $p < 0.01$, d.f. = 1) (Table 8). Most of the trenches in the Kuinderbos run NW – SE, but there is no preference for either aspect ($\chi^2 = 0.8$, $p > 0.1$). Hence there is a difference between the distribution of the colonies in the Kuinderbos compared with elsewhere in the Netherlands ($\chi^2 = 99.1$, $p < 0.001$, d.f = 3).

Composition of the vegetation.—Gymnocarpium dryopteris co-occurs with 97 higher plants and 38 mosses and liverworts (n = 37 relevés). Several clusters are discernable in the synoptic table (Table 9). In general, the clusters do not fit precisely into one community. Group I comprises wall vegetations. Among the species associated with *G. dryopteris* are *Dryopteris filix-mas* (L.) Schott and *Thelyperis palustris* Schott. The vegetation of group I are related to the *Asplenietum trichomano-ruta-murariae*. Group II comprises vegetation

TABLE 7. Inclination of colonies on walls and in trenches in the Netherlands (Pleistocene area in particular). Slope per colony is based on the highest value.

	Pleistocene area		Kuinderbos	
Slope	Number of colonies	%	Number of colonies	%
< 10°	2	4.6	17	25.5
11–20°	5	11.7	2	3.0
21–30°	5	11.7	1	1.4
31–40°	2	4.6	5	7.4
41–50°	13	30.2	12	17.9
51–60°	3	7.0	20	29.9
61–70°	1	2.3	6	8.9
71–80°	0	0.0	4	6.0
81–90°	12	27.9	0	0.0
Total	*43*	*100*	*67*	*100*

TABLE 8. Aspect of sites in the Netherlands (Pleistocene area in particular) with slope > 10° and in the Kuinderbos.

	Pleistocene area		Kuinderbos	
Aspect	Number of colonies	%	Number of colonies	%
NW-NE	26	42.6	172	41.0
E or W	12	19.7	0	0.0
SW-SE	9	14.7	190	45.4
none	14	23.0	57	13.6
Total	*61*	*100*	*419*	*100*

with relatively many acidophile species. Some of the relevés were in stands of exotics. The relevés display a clear relationship with the *Quercion roboris-petreae* (and within this, with the *Fago-Quercetum* and also the *Betulo-Quercetum*). The relevés made under *Picea sitchensis* (Kuinderbos) also fall into this group. Group III comprises relevés in which species such as *Sorbus aucuparia* L., *Fagus sylvatica* (seedling or juvenile) and *Lonicera periclymenum* L. are differentiating. These relevés are clearly assignable to the *Fago-Quercetum*, actually being a damp form of this association with *Athyrium filix-femina* (L.) Roth and *Lysimachia vulgaris* L. Group IV comprises a single relevé which includes, among others, *Geranium robertianum* L., *Aegopodium podagraria* L., *Oxalis europaea* L. and *Conocephalum conicum* (L.) Dumort, which are assignable to the *Pruno-Fraxinetum*. Group V comprises relevés with relatively many grasses. The characteristic species for this group include, among others, *Festuca rubra* L., *Holcus lanatus* L., *Cerastium fontanum* Baumg. and *Taraxacum officinale* F.H.Wigg. These sites are of *G. dryopteris* on basalt dikes and in roadside trenches; the relevés contain many species that occur in the adjacent roadside verges. The most reliable species associated with *G. dryopteris* are *Athyrium filix-femina*, *Dryopteris dilatata* (Hoffm.) A.Gray, *Rubus fruticosus* L., with *Athyrium filix-femina* in particular being an indicator of potential sites of *G. dryopteris*.

Accompanying fern species were recorded for 128 colonies in total. The most common within the Dutch population are *Athyrium filix-femina*, *Dryopteris filix-mas*, *D. dilatata* and *D. carthusiana* (Vill.) H.P.Fuchs. Outside the Kuinderbos there are 14 fern species that occur as associated species: on average, 2.2 species colony^{-1}. The various uncommon species recorded as associated fern species on canal walls include *Gymnocarpium robertianum*, *Polystichum aculeatum* (L.) Roth and *Asplenium trichomanes* L. In the Kuinderbos, *G. dryopteris* is associated with 12 fern species (Table 10). Four of the five patches of *Phegopteris connectilis* (Michaux) Watt in the Kuinderbos are associated with *G. dryopteris*. In some localities outside the Kuinderbos, both species occur directly next to each other or in the same area. The frequencies of *G. dryopteris* in the Kuinderbos and in the rest of the Netherlands being associated with other fern species are significantly different ($\chi^2 = 13.1$, $p < 0.001$, d.f. = 4); this is primarily because of the high frequency of *Dryopteris carthusiana* in the Kuinderbos.

TABLE 9. Synoptic table based on relevés with *Gymnocarpium dryopteris*. The data have been classified on the basis of a TWINSPAN computation of 37 relevés, two of which were made in Germany (Teutoburgerwoud) and the rest in the Netherlands. The table does not show the mosses, liverworts and vascular plant species recorded only once. Tree and shrub layer were not included in computation. The table gives the number of relevés per relevé group in which the species in question was found. Exclusive characteristic species have been printed in grey. These are species that have only been recorded in a single species group, with a presence of 25% or more. I = wall vegetations, II = exotic stands, III = *Fago-Quercetum*, IV = *Pruno - Fraxinetum*, V = vegetation of dikes and ditch banks.

Relevé group	I	II	III	IV	V
Number of relevés	4	16	8	1	8
Gymnocarpium dryopteris	4	16	8	1	8
Asplenium ruta-muraria	2	-	-	-	-
Asplenium trichomanes	2	-	-	-	-
Thelypteris palustris	3	-	-	-	-
Sagina procumbens	2	-	-	-	-
Capsella bursa-pastoris	2	-	-	-	-
Rubus idaeus	-	7	-	-	-
Betula pendula	-	5	-	-	-
Ceratocapnos claviculata	-	5	-	-	-
Quercus robur	-	4	-	-	-
Cardamine pratensis	-	3	-	-	-
Viola palustris	-	2	-	-	-
Festuca ovina	-	2	-	-	-
Humulus lupulus	-	2	-	-	-
Eupatorium cannabinum	-	2	-	-	1
Chamanaerion angustifolium	-	6	-	-	1
Oxalis europaea	-	1	-	1	-
Lonicera periclymeum	-	-	6	-	-
Equisetum arvense	-	-	2	-	-
Luzula multiflora	-	-	2	-	-
Oxalis stricta	-	-	2	-	-
Cerastium fontanum	-	-	-	-	4
Taraxacum vulgare	-	-	-	-	6
Tussilago farfare	-	-	-	-	3
Lolium perenne	-	-	-	-	2
Athyrium filix-femina	2	7	8	1	1
Dryopteris filix-mas	4	4	3	1	2
Dryopteris dilatata	1	14	4	-	-
Rubus sp.	4	9	6	-	-
Epilobium sp.	1	1	-	-	1
Stellaria media	-	2	1	1	1
Urtica dioica	-	3	4	1	2
Agrostis cappilaris	-	3	6	-	1
Galium aparine	-	1	3	-	2
Lysimachia vulgaris	-	2	6	-	1
Hieracium laevigatum	-	2	2	-	1
Juncus effusus	-	4	1	-	1
Poa trivialis	-	2	1	-	2
Dryopteris carthusiana	-	2	2	-	1
Polypodium vulgare	1	1	-	-	-
Senecio vulgaris	1	-	-	-	1
Holcus mollis	-	5	3	-	-

TABLE 9. Continued.

Relevé group	I	II	III	IV	V
Deschampsia flexuosa	-	4	3	-	-
Sorbus aucuparia	-	1	6	-	-
Fagus sylvatica (j)	-	1	5	-	-
Galium saxatile	-	2	2	-	-
Molina coerulea	-	1	2	-	-
Rumex obtusifolius	-	1	1	-	-
Ajuga reptans	-	1	1	-	-
Phegopteris connectilis	-	1	-	-	1
Galium palustre	-	1	-	-	1
Heracleum sphondylium	-	1	-	-	1
Calamagrostis epigejos	-	2	-	-	2
Phragmites australis	-	3	-	-	2
Holcus lanatus	-	4	-	-	5
Ranunculus repens	-	-	2	1	-
Geranium robertianum	-	-	1	1	-
Anthryscus sylvestris	-	-	1	1	-
Angelica sylvestris	-	-	1	1	-
Festuca rubra	-	-	1	-	7
Potentilla erecta	-	-	1	-	1
Prunella vulgaris	-	-	2	-	1

Size of colonies.—Sixty nine percent of the colonies in the Netherlands have fewer than 100 fronds. Large specimens with over 1000 fronds are rare: in the period 1979–1981, this occurred in only a single patch (in the park of the Royal Het Loo palace). In this respect the distribution for the Kuinderbos (1990 situation) is very similar to that for the rest of the Netherlands (Table 11), though the Kuinderbos has relatively more colonies with more than 1000 fronds (maximum is 3000). Various populations in this woodland were monitored over 15 years (Bremer, 1994). In heavy shade, individuals may remain the same size for a long time. If the light increases suddenly (e.g., as a result of storm damage), the number of fronds may increase over a number of years, at a maximum rate of 400 fronds year^{-1}. Seventy five percent of the colonies in the Netherlands are smaller in extent than this annual increase. Young clones lack a clear shape. As they grow larger, patches become clearly discernable. They are often oval or round in shape. If the center dies, a fairy ring may arise. Along trenches or streams, the oval shape may become elongated. Old patches may break up, making it appear that several clones occur next to each other. This has seldom been recorded in the Netherlands, because the clones are relatively young and limited in extent.

In the Netherlands (outside the Kuinderbos), 74 clones were recorded in the research period (1979–1991). By 1992, seven (= 10%) had disappeared. Sites on walls are always small and vulnerable, but large patches, too, can decay in a couple of years. Eighty eight percent of the Dutch colonies whose age was known or could be estimated were no older than 20 years (n = 41). After long-

TABLE 10. Associated fern species (within a distance of approx. 3 m from the clone) in the Netherlands (Pleistocene area in particular) and in the Kuinderbos (n = number of clones) For chi-square analysis, species with a frequency < 5 were clustered in a single category in order to fulfil the requirements of this test.

Species	Pleistocene area (n = 63)		Kuinderbos (n = 65)	
	Number of colonies	%	Number of colonies	%
Dryopteris dilatata	40	63.5	50	76.9
Athyrium filix-femina	36	57.1	35	53.8
Dryopteris filix-mas	22	34.9	25	38.5
Dryopteris carthusiana	14	22.2	40	61.5
Asplenium trichomanes	4	6.3	1	1.5
Phegopteris connectilis	4	6.3	3	4.6
Blechnum spicant	4	6.3	-	
Polypodium vulgare	3	4.8	1	1.5
Thelypteris palustris	3	4.8	-	
Pteridium aquilinum	2	3.1	-	
Asplenium ruta-muraria	1	1.6	-	
Polystichum aculeatum	1	1.6	2	3.1
Gymnocarpium robertianum	1	1.6	1	1.5
Asplenium scolopendrium	1	1.6	4	6.1
Polystichum setiferum	-		2	3.1
Dryopteris affinis	-		1	1.5
Mean no. of species per clone	*2.2*		*2.5*	

term monitoring of four research plots in the Kuinderbos it was found that after 15 years, 43.7% of the population (n = 190) had disappeared (Bremer, 1994). The half-life varied from 3.5 to 4.6 years, with some colonies older than 30 years.

Fertile fronds generally occur in specimens with more than 50 fronds, except in the case of wall specimens, where, though fronds are few, they may be fertile. Usually, less than 20% of the fronds of large colonies are fertile. In some large colonies, more than 50% of the fronds were found to be fertile. In general, fertile fronds are longer than sterile fronds and extend beyond the other fronds.

Frond damage.—Gymnocarpium dryopteris is a deciduous species. Its fronds usually unfurl in early May, though they may appear at the end of April if the winter has been mild. The species is vulnerable to damage from late night frosts, as evidenced after the April frosts of 1991. Frost can also accelerate die-back in October, but even without frost, most fronds have died before November. Severe thunderstorms may also cause damage, as if bent, the fragile fronds will die. New fronds can be formed until September, though they are usually smaller. *G. dryopteris* rarely suffers pest damage. Sawfly caterpillars (*Symphyta*, indet.) are regularly found on the fronds of specimens in the Kuinderbos, but rarely do they destroy entire fronds. Similar pest damage has been found on populations elsewhere in the Netherlands. Slug damage is rare. *Gymnocarpium dryopteris* growing on dikes in small crevices between basalt rocks may be damaged by browsing livestock.

TABLE 11. The size of colonies of *Gymnocarpium* dryopteris, based on the number of fronds colony $^{-1}$ n = number or colonies, % percentage of colonies. **a**. Dutch populations 1979–1991 (Pleistocene area in particular), **b.** population in Kuinderbos in 1979 52 48′N, 5 45′E, **c.** population in Kuinderbos in 1990, **d.** Harz, Bremketal (1989), in *Luzulo-Fagetum dryopteridetosum* 51 40′N 10 20′E , **e.** Weserbergland (1989), in Steinköpfe ancient woodland in *Fago – Quercetum*, 51° 28′N, 9° 20′E, **f.** Allgäu (1991) in *Abieti-Fagetum* 47°26′ N 10°25′E.

	Netherlands											
	Pleistocene area		Kuinderbos				Germany					
	a		b		c		d		e		f	
Size	n	%	n	%	n	%	n	%	n	%	n	%
1–10	11	17.2	35	17.2	19	10.9	1	1.8	-		2	14.2
11–100	29	45.3	124	60.8	75	43.1	13	24.1	5	12.8	6	42.9
101–1000	23	35.9	45	22.0	72	41.4	18	33.3	18	46.2	6	42.9
1001–10000	1	1.6	-		8	4.6	15	27.8	14	35.9	-	-
10001–10^5	-		-		-		4	7.4	2	5.1	-	-
10^5–10^6	-		-		-		3	5.6	-		-	-
Total	*64*	*100*	*204*	*100*	*174*	*100*	*54*	*100*	*39*	*100*	*14*	*100*

DISCUSSION

Gymnocarpium dryopteris is a very rare species in the Netherlands (van der Meijden, 2005). In the period 1979–1991, 670 clones were recorded. The only high density recorded was in the Kuinderbos. Here, the fern was closely associated with *Picea sitchensis* in 1979, whether the ecto-organic layer was overlying fine sand, peat or clay. The rhizome is frequently very shallow, and thus the species reacts strongly to the composition of the ecto-organic layer, which is determined by the dominant tree species. It is striking that all the sites have peat in the subsoil. This peat deposit supplies the uppermost deposits with water by capillary action, assuring a very stable moisture supply. In long droughts, like the one that occurred from May to August 1992, many specimens may display reduced vitality, but there is no massive die-back of fronds. The reason that rhizomes on trench banks are shallower than in the woodland may be that the moisture supply is better than in the adjacent woodland. Here, clones often occur almost as far as the bottom of the trench. In the Kuinderbos, most colonies are growing within the influence of the groundwater; elsewhere they also occur outside this sphere of influence. The species can thus be designated a local phreatophyte (Londo, 1988). Although it does not tolerate waterlogging in the root zone (Britten, undated), parts of clones will tolerate brief submergence. In the Netherlands the rooting depth varies from 0.5–8 cm, which is more than the 2–3 cm mentioned by Page (1982). The mean value does agree with Page (1982).

In the Netherlands (excluding Kuinderbos) *G. dryopteris* grows on acidic sites. Exceptions are the sites on canal walls, where the mortar has a high pH. In the Kuinderbos the pH is generally high, but this could be an artifact of the

soil sampling, as the ecto-organic layer is thin and when the root environment is being sampled, some of the underlying soil was also sampled. Clones growing on the transition from trench side to woodland floor may be growing in calcareous fine sand in the trench side, but in an acidic fermentation layer on the woodland floor. Hence within a single clone, the pH of the root zone may vary by several units. Page (1982) notes that in the United Kingdom, *G. dryopteris* can grow in various pH values. Dostál (1984) reported the species on limestone overlain by a layer of raw humus, on soils with a mull or a moder profile. In North America the species prefers weakly acid substrates (Lellinger, 1985). Ellenberg (1982) called the species a moder humus indicator, because it grows on litter under *Picea abies* (L.) H.Karst., for example.

Gymnocarpium dryopteris occurs in various woodland types, such as the *Fago-Quercetum* and *Pruno-Fraxinetum*, always in relatively damp places and often associated with *Athyrium filix-femina*. The data presented by Stortelder *et al.* (1999) are in agreement with this. Outside the Kuinderbos, stands of *Pseudotsuga mensiezii* are an important habitat. These have often been planted in places where the potential woodland type is the *Fago-Quercetum*. Sissingh (1970) goes so far as to call it a *Dryopterido-Pseudotsugetum* with, among others, acidophile mosses and various ferns, including *G. dryopteris* as characteristic species. *Gymnocarpium dryopteris* is found in natural Douglas fir forests in N. America (Snyder, 1993). The only localities in the Netherlands where the species has been observed in stands of *Picea sitchensis* are in East Flevoland and the Kuinderbos – especially the latter. On the west coast of the United States and Canada the species frequently occurs in the natural Sitka forests (Fonda 1974) and is common in the *Polystichum munitum - Picea sitchensis* association, which has been described as a closed coniferous forest thriving on the foggy and rainy coasts of British Columbia. It is also common in other associations of the montane and subalpine forests e.g. the *G. dryopteris - Abies amabilis* community (Barbour and Billings, 1988; Peinado *et al.*, 1997). The species has not been reported from the extensive plantations of *Picea sitchensis* in Scotland (Ford *et al.*, 1979; Peterken, 1985), or Wales (Hill and Jones, 1978), but can be expected in places where moisture is guaranteed.

After 1985 it was found that *G. dryopteris* could rapidly establish in many places in the Kuinderbos where very dense stands of *Picea abies* had been severely thinned. This suggests it is not strictly associated with *Picea sitchensis*. If the Kuinderbos had been planted with *Pseudotsuga mensiezii* rather than *Picea sitchensis*, similar densities could have been expected, given the similarities between both conifers in terms of the light climate and build-up of the ecto-organic layer. The colonization of thinned stands of *Picea abies* is in line with the behavior of the species in Central Europe. In southern Germany, *G. dryopteris* has been reported in 31 forest types (out of a total of 68); in eight forest types its frequency was more than 20%. The woodlands in question were in ravines (*Ulmo glabrae-Aceretum pseudoplatani*), or were beech (such as *Dentario-Fagetum* and *Aceri-Fagetum*) and *Picea abies* forests (such as *Asplenio-Piceetum*) (Oberdorfer, 1992). In the Harz mountains, it occurs in all types of beech forest, in subassociations in shady places with a

high relative humidity (Böttcher *et al.*, 1981). The center of the species' distribution in the United Kingdom is in the birch and oak woodlands in Scotland (Page,1982), but it also occurs along brooks with woodland vegetation related to the *Pruno-Fraxinetum* (Tansley, 1965). Rodwell (1991) calls it from *Juniperus communis - Oxalis acetosella* woodland with *Betula* spp. in the tree layer. In Scandinavia, the species is common in spruce, pine and birch forests (Aune, 1982; Havas and Kubin, 1985), and also in *Carpinus betulus* forests (Mäkirinta, 1982). In the farthest north it also occurs outside woodland, in grasslands (Westhoff and Schoof-van Pelt, 1982). Its occurrence in grazed vegetations on basalt dikes in the Netherlands is thus not unexpected, as this vegetation too is unshaded. In North America it is listed from 38 cover types, with a dominant role of types dominated by coniferous tree species (Snyder, 1993).

Gymnocarpium dryopteris is often associated with *Phegopteris connectilis* within its range. In the Netherlands *Phegopteris connectilis* co-occurs with *G. dryopteris* in seven places, and in the Kuinderbos, they often co-occur. It has been reported (Rasbach and Willmans, 1976) that in Central Europe *G. dryopteris* establishes on weathering chalk soils earlier than *Phegopteris connectilis* (Rasbach and Willmans, 1976) especially if an acid layer has formed on top of these soils. Page (1982) mentions that the ecological difference is that *Phegopteris connectilis* grows on slightly drier slopes. The ecological differences appear to be minor. It remains noteworthy that in the Netherlands and elsewhere, *Phegopteris connectilis* is much rarer than *G. dryopteris*. In the 1980s, the Dutch population of *G. dryopteris* was at least 670 clones, with approximately 80,000 fronds, compared with an estimated maximum of 50 clones of *Phegopteris connectilis*. In its favored sites (montane areas), more than 100,000 fronds may occur within 1 ha, as has been recorded in the Harz mountains (Germany). Colonies with more than 1000 fronds form 2% of the population in the Netherlands, 5% of the population in the Kuinderbos and 41% in the studied populations in the Harz mountains and Weserbergland. Although clones can increase their size rapidly if conditions are favorable, in the Netherlands they remain small.

Eighty eight percent of the Dutch colonies are less than 20 years old, which suggests many recent establishings, but also high mortality. Colonies tens of years old are rare. The species used to occur in Beekbergerwoud, before this last surviving ancient Dutch old growth was felled in 1871. The oldest existing sites are in the Royal park of Het Loo palace, where the species was first recorded in 1853. It is possible that the same clones are still present, which must therefore be at least 150 years old. In the Kuinderbos the mortality is related to the lack of light that causes clones to gradually diminish in size and to disappear after some years (Bremer, 1994). Elsewhere in the Netherlands, colonies have disappeared for reasons such as the destruction of biotopes, heavy trampling and structural depletion of groundwater. Colonies mostly remain discernable as clearly delimited patches. Oinonen (1971) established that in Finland, colonies were still discernable after 450 years. Only in ancient, undisturbed forests do such clones break up and become indistinguishable.

Peterken (1985) mentioned this phenomenon in rhizome geophytes in old deciduous woodland. In one of the most undisturbed ancient forests in Europe, the Bialowieza forest (Polen), *G. dryopteris* has been observed to have a very dispersed manner of growth that is interrupted only where the rootballs of windthrown trees have created a new habitat and there has been a concentration in the fronds per colony.

The final conclusion is that the population in the Kuiderbos differs from that of the rest of the Netherlands in many aspects. The high density of clones in this area makes it possible to identify which factors are important. These are: a good, constant supply of moisture from underground peat, the presence of an acid ecto-organic layer with both a litter layer and a fermentation layer, and a favourable light climate. These conditions can be met in moderately dense *Picea* stands. Although in the Kuinderbos the species is closely associated with Sitka spruce, *Picea sitchensis*, its occurrence elsewhere in the Netherlands under Douglas fir *Pseudotsuga menziesii* and the colonisation of *Picea abies* within the Kuinderbos indicate that moisture supply and the presence of the acid ecto-organic layer of Spruce needles are more important than the presence of *Picea sitchensis* in particular.

Acknowledgments

Thanks are due to the 26 respondents who completed questionnaires or supplied other information, to J. Burrough for translating the paper and F. Berendse for his comment on an earlier draft of the paper. I also thank two referees for valuable suggestions and comments.

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American Fern Journal 100(2):126–136 (2010)

Fungal Root Endophyte Colonization of Fern and Lycophyte Species from the Celaque National Park in Honduras

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Abstract.—Root endophyte colonization was investigated in 32 fern and lycophyte species of 11 families collected from the Celaque National Park in Honduras. Arbuscular mycorrhizae (AM) were found in 11 plant species (34%) of Anemiaceae, Gleicheniaceae, Ophioglossaceae, Pteridaceae, Selaginellaceae, Thelypteridaceae, and Woodsiaceae. The abundance of arbuscular mycorrhizal fungi (AMF) in roots varied with particular species, ranging from 4% (*Sticherus underwoodianus*) to 78% (*Thelypteris patens*). The morphological AM colonization pattern of all investigated species was of the *Paris*-type. The mycelium of dark septate endophytes (DSE) was found in 19 species (58%), and was observed both in the roots of plants that were colonized by AMF and were devoid of AM association. However, in both cases the percentage of root colonization by these fungi was low. Exceptions were *Asplenium serra*, *Elaphoglossum erinaceum*, *Lellingeria prionodes*, and *Lycopodium thyoides*, where abundant DSE hyphae were observed. Our results are the first detailed report of both AMF and DSE associations of these plant species. Moreover, the mycorrhizal status of 27 plant species is reported for the first time.

Key Words.—arbuscular mycorrhizae (AM), arbuscular mycorrhizal fungi (AMF), AM morphology, dark septate endophytes (DSE), ferns, *Paris*-type

Mycorrhizal symbiosis is the most widespread and commonly studied type of plant symbiotic associations. Nevertheless, the knowledge of mycorrhizae of ferns and lycophytes is scarce as only ca 10% of known species has been studied regarding their colonization by symbiotic fungi, namely arbuscular mycorrhizal fungi (AMF) (Glomeromycota) (Kessler *et al.*, 2009). There is a need to investigate mycorrhizae of ferns and lycophytes especially from tropical regions, where these groups of plants are most diverse and

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ecologically important, and where most major lineages of these plants have probably evolved (Kessler *et al.*, 2009, 2010). Moreover, little is known about associations of ferns and lycophytes with dark septate endophytes (DSE), a taxonomically and ecologically diverse group of fungi (Jumpponen, 2001). Among DSE, neutral, parasitic and symbiotic interactions with seed plants have been documented (Haselwandter and Read, 1982; Jumpponen, 2001; Wu and Guo, 2008); however, only few investigations have focused on their associations with ferns and lycophytes (Fernández *et al.*, 2008; Kessler *et al.*, 2009, 2010). In this study, we examined the fungal root endophyte colonization of fern and lycophyte species from the Celaque National Park in Honduras. We evaluated mycorrhizal status, the degree of AMF and DSE root colonization, and the morphotypes of arbuscular mycorrhiza (AM).

Materials and Methods

Study area and material sampling.—The plant material for fungal root endophyte colonization assessment was collected from the Celaque National Park in Honduras in March/April 2008. The specimens were collected at various sites between 1000 m and 2800 m above sea level. We usually sampled one specimen per species except for four fern species, of which more than one sample was collected from the same habitat. In total, 34 plant taxa (32 species and two specimens assigned only to the genus level) from 11 families were sampled (Table 1). Whole plants were excavated and cleaned mechanically from the substrate. The roots were cut from the specimens, washed in water and stored in 50% ethanol. The plant specimens were deposited in the herbaria KRA (Institute of Botany, Jagiellonian University, Kraków, Poland) and TEFH (Universidad Nacional Autónoma de Honduras, Tegucigalpa, Honduras).

Root staining and the assessment of fungal colonization.—Roots were prepared according to the method of Phillips and Hayman (1970), with modifications. Roots were cleared in 10% KOH for 24 h and then rinsed in water. If the roots were still dark, the KOH was changed and the material was kept for further 24 hours, up to 72 h. The material was then acidified in 5% lactic acid in water (24 h), stained with 0.05% aniline blue in 80% lactic acid (72 h), and finally stored in 80% lactic acid until analysis. The whole procedure was performed at room temperature (22 °C). Root fragments (ca 1 cm) were mounted on slides in glycerol:lactic acid (5:1) and squashed using coverslides. The number of root fragments analyzed in the case of each specimen is reported in Table 1.

Fungal root colonization (AMF and DSE) was assessed using Nikon Eclipse 80i light microscope with Nomarski interference contrast optics and a digital camera with a panel for image analysis. The AMF colonization was identified on the basis of aseptate hyphae of irregular diameter, growing (i) intercellularly, forming arbuscules terminally in cortical cells (*Arum*-type of AM morphology), (ii) intracellularly with arbuscules developed on coils (*Paris*-type) or (iii) forming intermediate types (Dodd *et al.*, 2000; Smith and Read,

TABLE 1. Fungal root endophytes of fern and lycophyte species collected from the Celaque National Park.

Family [a]	Plant species [b]	Specimen root no. [c]	Habitat [d]	AM literature status [e]	AM parameters [f] F	M	A	Fv	F_{DSE} [g]
Anemiaceae	*Anemia phyllitidis* (L.) Sw.	109 (30)	T	1+	100	77	76	24	12
Aspleniaceae	*Asplenium achilleifolium* (M. Martens & Galeotti) Liebm.	155 (30)	E	NS	-	-	-	-	7
	Asplenium auriculatum Sw.	153 (30)	E	NS	-	-	-	-	10
	Asplenium fragrans Sw.	117 (30)	E	NS	-	-	-	-	-
	A. fragrans	85 (30)	E		-	-	-	-	-
	Asplenium praemorsum Sw.	135 (30)	E	1,2-	-	-	-	-	-
	Asplenium serra Langsd. & Fisch.	156 (20)	E	NS	-	-	-	-	65
	Asplenium sp.	99 (30)	E	1+/−	-	-	-	-	43
Dryopteridaceae	*Elaphoglossum erinaceum* (Fée) T. Moore	76 (20)	E	NS	-	-	-	-	100
	Elaphoglossum setigerum (Sodiro) Diels	71 (10)	E	NS	-	-	-	-	-
Gleicheniaceae	*Diplopterygium bancroftii* (Hook.) A.R. Sm.	130 (15)	T	NS	47	12	4	20	100
	Sticherus fulvus (Desv.) Ching	113 (20)	T	NS	-	-	-	-	-
	Sticherus underwoodianus (Maxon) Nakai	126 (17)	T	NS	29	4	3	-	12
Lycopodiaceae	*Lycopodium thyoides* Humb. & Bonpl. ex Willd.	128 (10)	T	NS	-	-	-	-	100
Ophioglossaceae	*Botrychium virginicum* Willd.	147 (12)	T	1,3+	100	71	71	17	-
Polypodiaceae	*Campyloneurum amphostenon* (Kunze ex Klotzsch) Fée	75 (20)	E	NS	-	-	-	-	-
	Campyloneurum angustifolium (Sw.) Fée	102 (25)	E	NS	-	-	-	-	8
	C. angustifolium	149 (30)	E		-	-	-	-	17
	C. angustifolium	157 (20)	E		-	-	-	-	-
	Campyloneurum tenuipes Maxon	116 (20)	E	NS	-	-	-	-	9
	Lellingeria prionodes (Mickel & Beitel) A.R. Sm. & R.C. Moran	83 (22)	E	NS	-	-	-	-	32
	Niphidium crassifolium (L.) Lellinger	96 (20)	T	NS	-	-	-	-	9
	Pleopeltis mexicana (Fée) Mickel & Beitel	74 (30)	E	NS	-	-	-	-	-
	Pleopeltis polypodioides (L.) E.G. Andrews & Windham	136 (30)	E	NS	-	-	-	-	-
	Polypodium plebeium Schltdl. & Cham.	138 (20)	E	NS	-	-	-	-	-
	Polypodium pleurosorum Kunze ex Mett.	150 (30)	E	NS	-	-	-	-	-
	P. pleurosorum	81 (30)	E		-	-	-	-	-
	Polypodium subpetiolatum Hook.	77 (10)	E	NS	-	-	-	-	-
	Terpsichore sp.	72 (18)	E	NS	-	-	-	-	-

TABLE 1. Continued.

Family [a]	Plant species [b]	Specimen root no. [c]	Habitat [d]	AM literature status [e]	AM parameters [f]				
					F	M	A	Fv	F_{DSE} [g]
Pteridaceae	*Adiantum andicola* Liebm.	112 (10)	T	NS	80	53	53	10	20
	A. andicola	122 (10)	T		-	-	-	-	40
	A. andicola	78 (18)	T		100	73	67	-	-
	Chelianthes complanata A.R. Sm.	73 (16)	T	NS	69	37	36	13	13
	Pityrogramma ebenea (L.) Proctor	114 (6)	T	NS	-	-	-	-	60
Selaginellaceae	*Selaginella pallescens* (C. Presl) Spring	101 (22)	T	NS	63	11	4	64	14
Thelypteridaceae	*Macrothelypteris torresiana* (Gaudich.) Ching	95 (20)	T	1+, 2+/−	90	65	65	85	-
	Thelypteris patens (Sw.) Small	121 (23)	T	NS	100	78	76	26	17
	Thelypteris pilosula (Klotzsch & H. Karst. ex Mett.) R.M. Tryon	98 (15)	T	NS	-	-	-	-	-
Woodsiaceae	*Cystopteris fragilis* (L.) Bernh.	158 (16)	T	1,3+/−, 4+	100	68	61	50	38
	Diplazium franconis Liebm.	146 (22)	T	NS	55	11	10	-	9

[a] Plant family names according to Smith *et al.*, 2006.

[b] Plant species names mainly according to Nelson Sutherland *et al.*, 1996.

[c] Specimen number (Naks *et al.*). Number of root fragments (ca 1 cm) analyzed is given in parenthesis after the specimen number.

[d] Habitat: E – epiphytic, T – terrestrial.

[e] Arbuscular mycorrhizal (AM) status of investigated fern and lycophyte species according to available literature: 1 – Wang and Qiu, 2006; 2 – Zhao, 2000; 3 – Berch and Kendrick, 1982; 4 – Cooper, 1976; (+) AM present, (-) AM absent, NS – not surveyed.

[f] AM status observed in this study: mycorrhizal parameters [%]: F – mycorrhizal frequency, M – relative mycorrhizal root length, A – relative arbuscular richness; (-) AM fungi absent; Fv – frequency of the occurrence of vesicles, (-) vesicles absent.

[g] F_{DSE} – frequency of the occurrence of dark septate endophyte (DSE) mycelium [%]; (-) DSE absent.

2008; Zubek *et al.*, 2008; Zubek and Błaszkowski, 2009). The method proposed by Trouvelot *et al.* (1986) was followed for assessment of AM development. The parameters evaluated were: mycorrhizal frequency (F), relative mycorrhizal root length (M), and relative arbuscular richness (A). An estimate of mycorrhizal frequency (F%) is given as the ratio between root fragments colonized by AMF mycelium and the total number of root fragments analyzed. The relative mycorrhizal root length (M%) is an estimate of the amount of root cortex that is mycorrhizal relative to the whole root system. Arbuscule abundance (A%) is an estimate of arbuscule richness in the whole root system (Trouvelot *et al.*, 1986). DSE colonization was identified on the basis of regularly septate hyphae, usually dark pigmented, with facultatively occurring sclerotia (Jumpponen, 2001; Zubek *et al.*, 2008; Zubek and Błaszkowski, 2009). The frequency of DSE mycelium occurrence in roots (F_{DSE}%) was estimated as detailed above for AMF. Additionally, the frequency of the occurrence of resting spores of the fungi from the genus *Olpidium* (F_{Olp}%) was assessed (Zubek *et al.*, 2008; Zubek and Błaszkowski, 2009).

Results

AM status.—Arbuscular mycorrhizae with arbuscules, which are the structural and functional criterion of the symbiosis, were found in 11 species (34%) from the families Anemiaceae, Gleicheniaceae, Ophioglossaceae, Pteridaceae, Selaginellaceae, Thelypteridaceae, and Woodsiaceae. The abundance of AMF in roots varied widely with particular taxa (Table 1). The highest AMF colonization was found in *Thelypteris patens* (M=78%) and *Anemia phyllitidis* (M=77%), which were also characterized by high arbuscule richness (A=76%). The lowest AMF colonization and arbuscule abundance were observed in *Sticherus underwoodianus* (M=4%, A=3%). In the case of *Adiantum andicola*, two of three analyzed root samples were mycorrhizal (M=53% and 73%), and one was devoid of AMF (Table 1).

All plant species showed *Paris*-type colonization in which AMF colonized roots by intracellular growth. Neighboring cortical cells contained hyphal coils on which arbuscules were formed (Fig. 1).

DSE colonization.—DSE were found in 19 identified species (58%) and in the roots of *Asplenium* sp. (Table 1). DSE colonization was observed in the roots of plants which were either colonized by AMF (9 specimens) or were devoid of AMF (13 specimens). Although the frequency of DSE occurrence in roots was above 30% in several species, the percentage of root colonization was low (data not shown). Single hyphae of different diameters, accompanied sporadically by sclerotia (Fig. 2), were found in the outer cortex and rhizodermis. Exceptions were *Asplenium serra*, *Elaphoglossum erinaceum*, *Lellingeria prionodes*, and *Lycopodium thyoides*, where DSE hyphae occurred abundantly in most analyzed root fragments (Fig. 2). DSE colonized up to 80% of root cortex of these plants. The mycelium was brownish or stained with aniline blue.

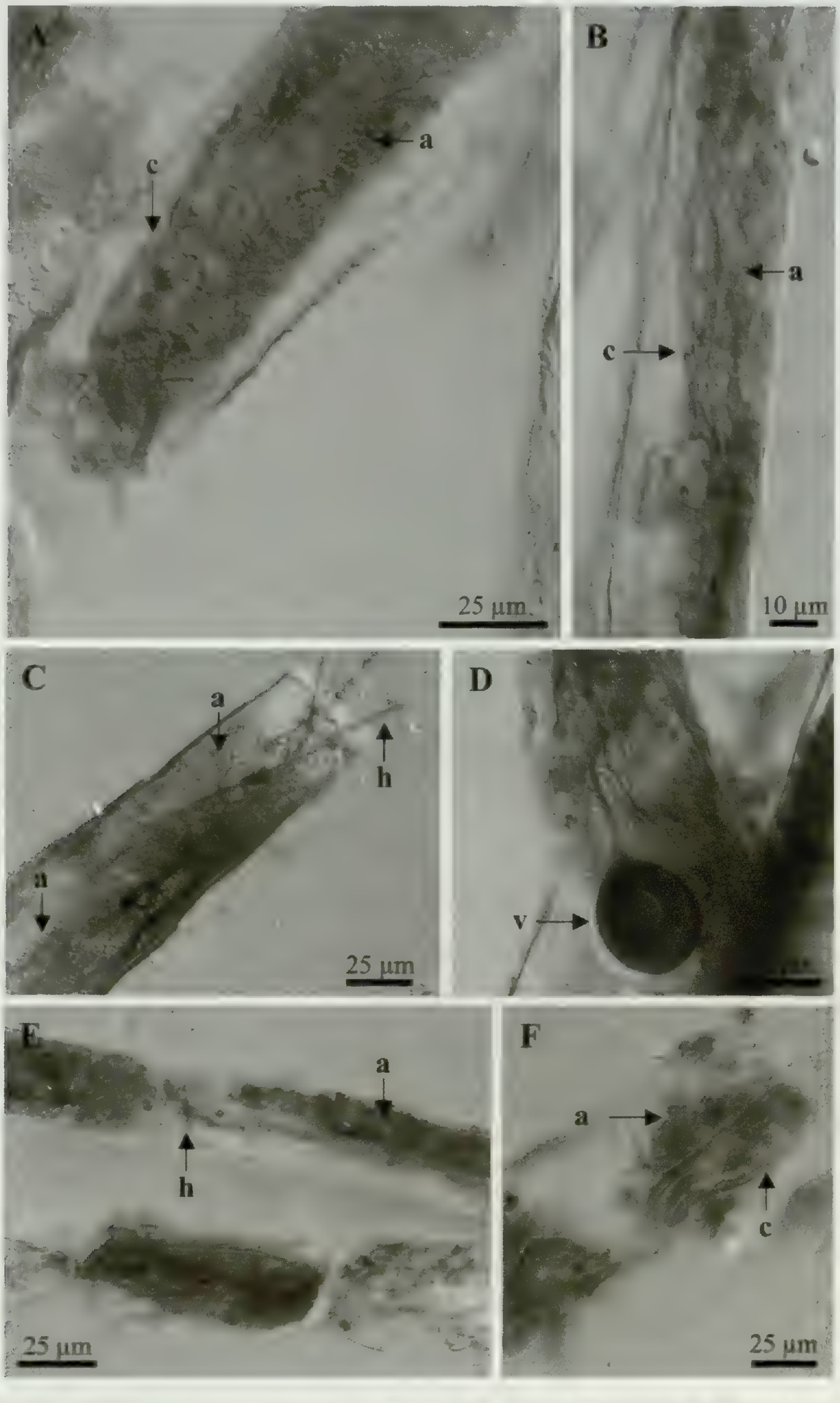

FIG. 1. A–F. Light micrographs of AMF mycelium in the root of *Cystopteris fragilis* (A, B), *Anemia phyllitidis* (C, D), and *Macrothelypteris torresiana* (E, F) (*Paris*-type); h – AMF hyphae growing intracellularly from cell to cell, a – arbuscules formed on coils, c – coils, v – vesicle. In the case of micrographs D and F, the AMF mycelium fell out the cells due to root squashing on slides.

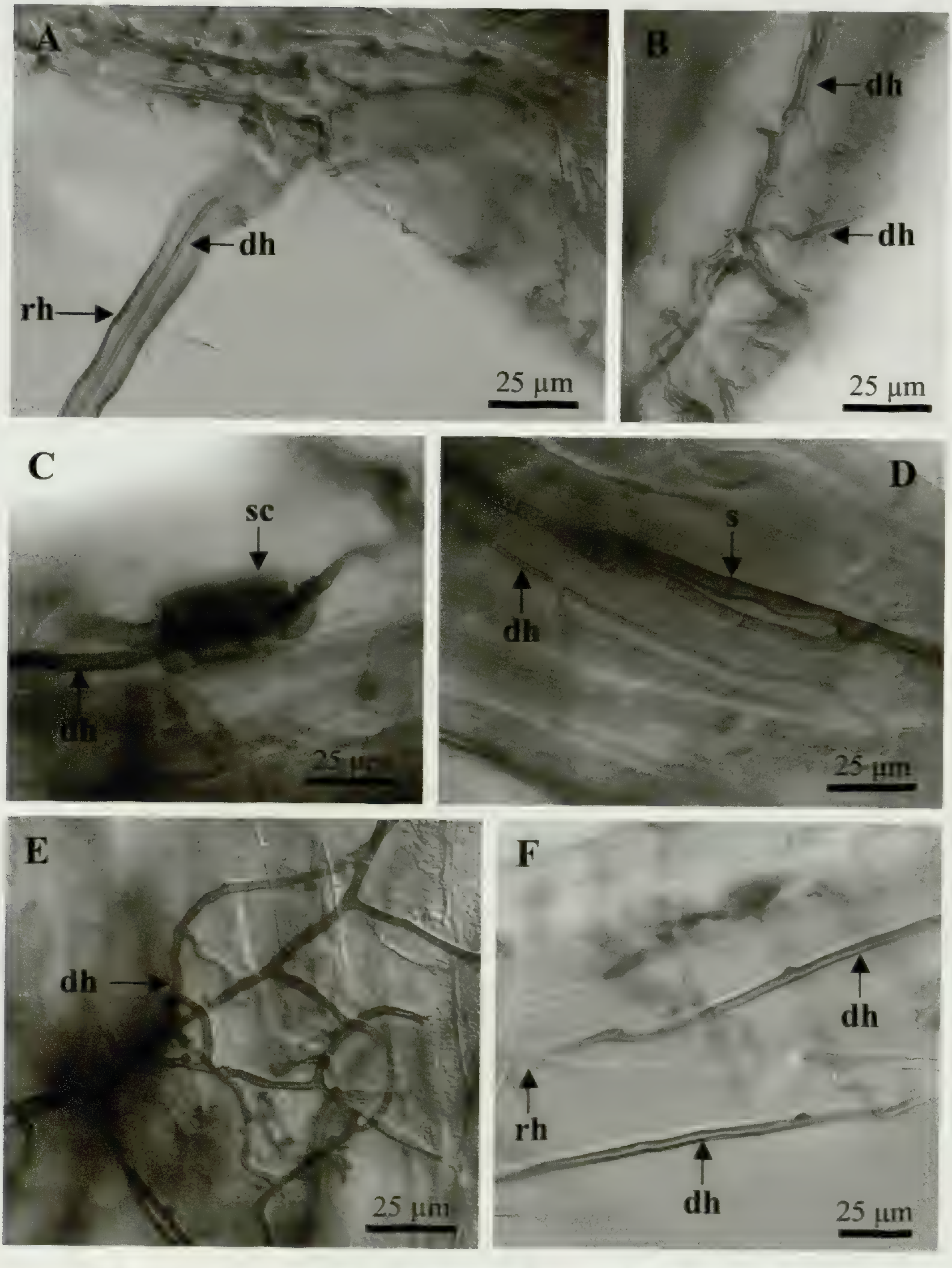

FIG. 2. A–F. Light micrographs of DSE mycelium observed in the root of *Elaphoglossum erinaceum* (A, B), *Adiantum andicola* (C), *Lycopodium thyoides* (D), and on the root surface of *Anemia phyllitidis* (E) and *Selaginella pallescens* (F); dh – DSE hyphae, sc – sclerotium, s – septa, rh – root hairs.

Single DSE hyphae were also detected on the root surface and/or between root hairs of 28 specimens (Fig. 2). In the old roots of several species, which were not included in the assessments of AMF and DSE colonization, DSE mycelium was abundant.

Other fungal endophytes.—From other fungal root endophytes, only *Polypodium pleurosorum* had resting spores of *Olpidium* spp., which were stained with aniline blue. Both the frequency of these structures (F=20%) and the percentage of root infection were low.

Discussion

In this paper, we present the detailed report of AMF and DSE associations in 32 fern and lycophyte species. The mycorrhizal status of 27 species is reported, to the best of our knowledge, for the first time. The presence of AMF in *Anemia phyllitidis* and *Botrychium virginicum*, as well as the absence of AMF in *Asplenium praemorsum* were confirmed, consistent with previous literature (Berch and Kendrick, 1982; Zhao, 2000; Wang and Qiu, 2006). *Cystopteris fragilis* and *Macrothelypteris torresiana* were highly colonized by AMF, being reported earlier as either mycorrhizal or devoid of AMF (Cooper, 1976; Berch and Kendrick, 1982; Zhao, 2000; Wang and Qiu, 2006). An obvious shortcoming of our study is the limitation of our investigations, in the case of most species, to one individual per species. Thus, our observations that particular species lack mycorrhizae do not mean that they are devoid of AMF at other sites and habitats. In order to clarify if a species lacks the symbiosis, or is facultatively or obligatory mycorrhizal, the collection of repetition samples from the same and different habitats is needed. Nevertheless, our investigations add data to the knowledge of mycorrhizal status and the biology and ecology of several fern and lycophyte species. Furthermore, as it was recognized for endangered seed plant species, studies on mycorrhizal associations may also be important in the context of ecological restoration, preservation, and propagation (Barroetavena *et al.*, 1998; Turnau and Haselwandter, 2002; Fuchs and Haselwandter, 2004; Zubek *et al.*, 2008, 2009).

Investigations on the occurrence of mycorrhizae in ferns and lycophytes indicate a considerable diversity of interactions, ranging from lack of symbiosis to facultative and obligate associations (Zhao, 2000; Turnau *et al.*, 2005; Fernández *et al.*, 2008; Kessler *et al.*, 2009, 2010). The positive response of ferns to AMF was confirmed in experimental research. In the studies carried out on *Pellaea viridis* (Forsk.) Prantl (Pellaeaceae), the presence of AMF in both gametophytes and sporophytes resulted in larger leaf area and root length of sporophytes in comparison to the plants devoid of AMF (Turnau *et al.*, 2005). As suggested earlier, the formation of mycorrhizae might be of great importance for epiphytic plants due to nutrient shortages under these conditions (Kessler *et al.*, 2009). However, we observed that all epiphytes were devoid of AMF. Although this survey, due to aforementioned limitations in specimen collection, does not allow definite conclusions about the relationship between AM presence and habitats, our findings are in

accordance with previous studies on ferns and angiosperms, where low AMF colonization or the absence of AMF were found among epiphytes (Lesica and Antibus, 1990; Maffia *et al.*, 1993; Michelsen, 1993; Kessler *et al.*, 2009, 2010). In this habitat, unsuitable growth conditions for AMF (the lack of well-developed soils) seem to be the reason for the absence or low AM colonization rather than a lack of functional need among epiphytes for mycorrhizal associations (Kessler *et al.*, 2009).

It is known that the AM morphology may depend on the host plant and fungal identity, and that different environmental factors may have an impact on AM pattern of root colonization (Cavagnaro *et al.*, 2001; Dickson, 2004; Yamato, 2004; Kubota *et al.*, 2005; Dickson *et al.*, 2007; Smith and Read, 2008). In the present study, all mycorrhizal plant species showed *Paris*-type of colonization. The results are in accordance with other studies where mostly this morphotype has been found among ferns and lycophytes (Zhang *et al.*, 2004; Dickson *et al.*, 2007). However, intermediate types, and less frequently *Arum* morphology of AM have also been documented (Dickson *et al.*, 2007; Fernández *et al.*, 2008; Kessler *et al.*, 2009). Our observations confirm earlier records that the Gleicheniaceae, Ophioglossaceae, Pteridaceae and Woodsiaceae (the genera *Cystopteris* and *Diplazium* included in the Athyriaceae family by Zhang *et al.*, 2004) only have the *Paris*-type. However, both intermediate and *Paris* patterns of AM colonization have been observed among species of the families Selaginellaceae and Thelypteridaceae (Zhang *et al.*, 2004; Dickson *et al.*, 2007). To the best of our knowledge, the AM morphology is reported for the first time for all investigated species and for the Anemiaceae family.

Dark septate endophytes (DSE) were found in the roots of several species, which is in accordance with earlier observations on ferns and lycophytes (Fernández *et al.*, 2008; Kessler *et al.*, 2009, 2010). DSE represent a taxonomically and ecologically diverse group of fungi. Among them, neutral, parasitic and symbiotic interactions with plants are known (Jumpponen, 2001). In the case of our study, it is difficult to judge the character of the DSE associations with the investigated species. Nevertheless, in the case of single DSE hyphae presence in a root system it seems unlikely that the fungus would have a significant impact on a plant. However, DSE hyphae occurred abundantly in the roots of *Asplenium serra*, *Elaphoglossum erinaceum*, *Lellingeria prionodes*, and *Lycopodium thyoides*, and an important influence of DSE on their hosts can not be excluded.

A positive response of angiosperms to DSE has been found in a few studies. DSE isolates were shown to stimulate plant growth or increase phosphorus concentration in two *Carex* species (Haselwandter and Read, 1982). DSE were also found to colonize roots of *Saussurea involucrata* Kar. et Kir. ex Maxim (Asteraceae). In a laboratory experiment, seedlings of this species were inoculated with a DSE strain previously isolated from *S. involucrata* roots; the plants were colonized heavily and displayed enhanced growth due to the presence of DSE fungus (Wu and Guo, 2008). In order to reveal the nature of DSE associations with ferns and lycophytes, further research is necessary under experimental conditions. To our knowledge, such studies have not been conducted so far.

Acknowledgments

We are grateful to Administracion Forestal Del Estado Coorporacion Hondureña De Desarrollo Forestal (AFE-COHDEFOR) for the permission (2/GG-032-2008) for plant material collection. The present research was financially supported by the Jagiellonian University, projects no. DPB/SPK-414/CRBW/27/2007; K/ZBW/000008 and WB/B/DS/764/2008; K/ZDS/000003.

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The "American Fern Journal" (ISSN 0002-8444) is an illustrated quarterly devoted to the general study of ferns. It is owned by the American Fern Society, and published at The American Fern Society, % Missouri Botanical Garden, P. O. Box 299, St. Louis, MO 63166-0299. Periodicals postage paid at St. Louis, MO, and additional entry.

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American Fern Journal 100(3):137–147 (2010)

Ecological Responses of *Osmunda regalis* to Forest Canopy Cover and Grazing

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ABSTRACT.—The density and frond traits of the dimorphic fern *Osmunda regalis* were examined in 45 populations situated in central Italy, along a gradient of forest canopy cover and deer grazing. The survey involved 1704 ramets containing 6416 fronds, of which 5643 were sterile and 773 fertile. Within the ramets, the number of fertile fronds increased with the number of sterile fronds and with the mean height of ramets. Light seemed to promote fertility, as the number of fertile fronds decreased with increasing canopy cover. Fewer and smaller fronds were found in plots with high forest canopy cover. Our results showed that *O. regalis* performed best in conditions of full sunlight but can persist and recruit young sporophytes under closed canopy. As many marsh and lake environments have disappeared or been altered by human activity, more populations were located under high canopy cover in the forest than in open habitats. Deer grazing caused a significant decrease in fertile fronds, in total frond area and in ramet height, but the number of ramets and sterile fronds were unaffected. In conclusion, plant density and frond traits of *O. regalis* responded differently to the availability of light and to grazing.

KEY WORDS.—deer, fertile fronds, fern, Italy, populations, ramets, sporophyte, sterile fronds

Research on the effects of light on the density and frond traits of fern sporophytes can reveal important aspects of fern ecology and their role in forests, and may be a useful means of protecting endangered species. According to a recent critical review (Mehltreter, 2008), few researchers have contributed to this branch of ecological studies and the results are consequently restricted to a limited number of sites, which are usually located in the tropics as the majority of ferns are found there. Moreover, while many investigations have been carried out on tree-like ferns or tree ferns, especially due to commercial interest in many of them (i.e., Williams-Linera, 1997; Arens and Sánchez-Baracaldo, 2000; Arens, 2001; Chiou *et al.*, 2001; Mehltreter and García-Franco, 2008; Eleutério, 2009), few studies have examined herbaceous ferns.

An overview of research concerning the effect of light availability on the density and frond traits of herbaceous ferns showed that: frond density in *Dennstaedtia punctilobula* (Michx.) Moore increases with the intensity of light (Hammen, 1993); the sporophylls of *Matteuccia struthiopteris* (L.) Tod. increase with increased sunlight (Prange and von Aderkas; 1985, Odland *et al.*, 2006); the specific leaf area of various *Blechnum* species decreases with the increasing availability of light (Saldaña *et al.*, 2005); the rate of leaf

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development to maturity in *Rumohra adiantiformis* (Forst.) Ching is strongly related to solar radiation (Strandberg, 2003); stem growth rates and the percentage of stems with sori in *Oleandra pistillaris* (Sw.) C. Chr. were higher in the open than in the understorey (Takahashi and Mikami, 2006). Furthermore, research by Kluthe (2006) underlined that some selected fern species (*Athyrium filix-femina* "Frizelliae", *A. niponicum* "Pictum", *Dryopteris celsa* (W. Palmer) Knowlt., *D. erythrosora* (D.C. Eaton) O. Kuntze and *Matteuccia struthiopteris*) were significantly larger in 80% shade treatments than in 50% shade treatments, in relation to one or more of the parameters measured (total area, length or width of fronds).

It has been observed that the utilization of leaf primordia in *Osmunda cinnamomea* L. increased considerably following the removal of canopy and the consequent increase in light intensity (Jordan and Kuehnert, 1975). Nevertheless, light availability can have limited impact on the distribution, abundance and morphological parameters of other fern species (see Hill and Silander, 2001; Kluthe, 2006). Studies on the developmental stages of sporophytes have shown that light intensity may have different effects on mature and sporeling stages within the same species (Cinquemani Kuehn and Leopold, 1993). Other studies have shown correlations between frond size, growth and fertility and various environmental factors (i.e., temperature, water characteristics, etc.) (Odland, 1995; Vöge, 1997a; Vöge, 1997b; Odland, 1998; Odland et al., 2004).

Research into the effects of deer grazing on fern density and frond traits is scarce. Unlike spermatophytes, pteridophytes are less favored by and often unpalatable to deer (Nugent, 1990; Kraus, 1992; Suzuki *et al.*, 2008). However, studies carried out at the species level have pointed out that deer include some ferns among the favorite plants in their diet (see Forsyth *et al.*, 2002; 2005). This preference may be a result of seasonal variation, as ferns are important in the diet during late autumn, winter and early spring when they are one of the few sources of green leaves (Healy, 1971).

The goal of this study was to analyze whether and how a forest canopy cover gradient and deer grazing affect density and frond traits in some natural populations of royal fern (*Osmunda regalis* L. subsp. *regalis*). Analyses were performed at ramet level to investigate the relationship between traits, and at plot level to test the effect of canopy cover and grazing.

Osmunda regalis is a deciduous herbaceous fern with sterile fronds (trophophylls) and fertile fronds (trophosphorophylls), the latter bearing sporangia at their apex. The research was conducted in Tuscany (central Italy) where the species occurrence is mainly restricted to small, undisturbed habitat patches that offer the most favorable environmental conditions (see Landi and Angiolini, 2008). The worldwide distribution of *O. regalis* is connected to areas with sub-oceanic climates and the species has not adapted to either continental or dry climates (Pichi Sermolli, 1970; 1979). For this reason it is common in western Europe, where the Mediterranean regions (i.e., Spain and Italy) represent the southern limit of its distribution (Jalas and Suominen, 1972). In Italy the species is only present along the Alpine arc and in the

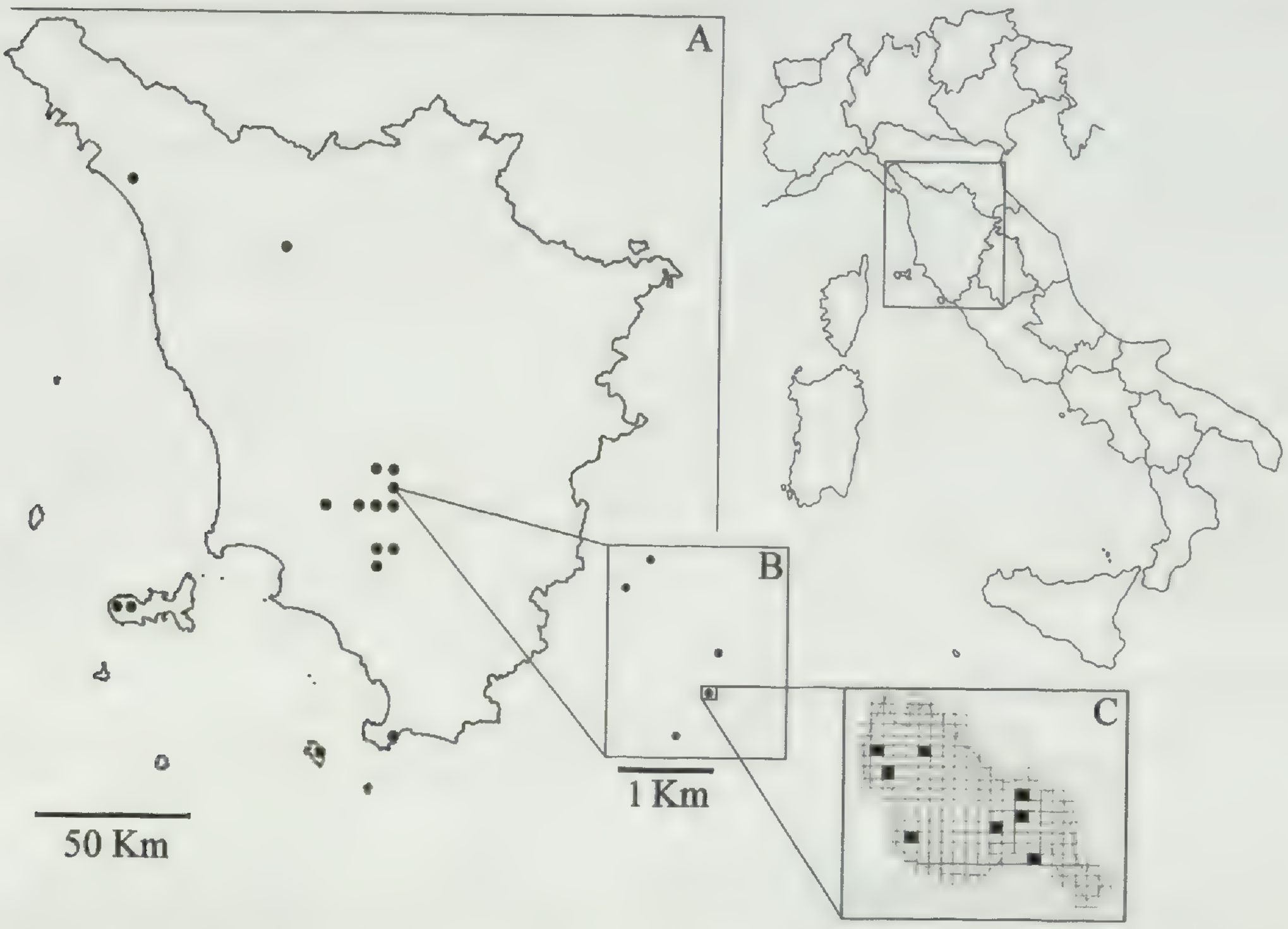

FIG. 1. Location map of the study area and sites investigated, showing dots representing 5 × 5 km grids (A), an example of the population distribution (B), and a representation of the random sampling design in a population (C); the black filled squares of 5 × 5 m indicate the plot sampled.

Tyrrhenian regions (Bizzarri, 1963; Marchetti, 2003) where it is often considered rare (see Camoletto Pasin *et al.*, 2000; Landi and Angiolini, 2007). The rarity of *O. regalis* in Tuscany is related not only to its geographically marginal position but also to the reduction or alteration (reclamation and water catchment) of the habitats in which it grows (Landi and Angiolini, 2007). The royal fern is also catalogued as rare and threatened in some regions of Spain where it is employed for medicinal uses and its harvesting is not strictly regulated (Molina *et al.*, 2009), and has decreased significantly in many other countries of Europe (Tutin *et al.*, 1993).

MATERIALS AND METHODS

The study was conducted between June and August 2006 on 45 natural populations of *Osmunda regalis* (*Osmundaceae*) located in Tuscany (central Italy, Fig. 1), at an altitude ranging between 5 and 620 m a.s.l.. Many of the larger populations occur at altitudes between 300 and 400m and with northeastern exposure (Landi and Angiolini, 2008). The majority of known *O. regalis* sites were investigated in this study, in order to cover all habitat types. The habitats occupied by these populations are swamps developing at or along the outflow of springs, the edge of streams and small marshes. The

region investigated is characterized by a Mediterranean climate. According to the meteorological stations (15 stations) closest to the populations, the mean annual precipitation is 916 mm/yr (±211 S.D.) and the mean annual temperature is 14.7 °C (±1.3 S.D.). The vegetation surrounding the populations is mainly composed of deciduous forests (Landi and Angiolini, 2010), and the tree species found in the fern populations (from most to least frequent, with the exception of rare species) are: *Fraxinus ornus* L., *Alnus glutinosa* (L.) Gaertn., *Castanea sativa* Mill., *Ilex aquifolium* L., *Quercus ilex* L., *Sorbus torminalis* (L.) Crantz, *Frangula alnus* Mill., *Arbutus unedo* L., *Carpinus betulus* L. and *Populus tremula* L.. The substrate of the sites is quite homogeneous and includes metamorphic units, magmatic rocks and quaternary deposits, with generally acidic or subacidic reaction. The only deer species present are fallow deer (*Dama dama* Linnaeus, 1758) and roe deer (*Capreolus capreolus* Linnaeus, 1758). A 2006 census by the Faunal Resources and Nature Reserve Service of the Province of Siena revealed that the deer density in the Merse Valley area, where *O. regalis* is most abundant (see Landi and Angiolini, 2008), is estimated at 12 animals/100 ha.

The *O. regalis* populations were defined as discrete clusters of plants, separated from other clusters by at least 500 m (Fig. 1b). Each population area was defined as a regular-shaped polygon covering the bulk of the population and 5 m × 5 m plots were randomly selected on a grid in order to sample 5% of the population (Fig 1c). Then, in order to obtain a representative sample of each population, a number of plots was selected in proportion to the surface area occupied by each population. For each ramet (a ramet being one but usually more fronds growing out of the rootstock like a crown, see Kenkel, 1996; Landi and Angiolini, 2008) the following attributes were recorded: number of fertile and sterile fronds, height, size of the fronds expressed as area (length × mean width) and presence of grazing damage on each frond. For each of 128 plots investigated the following parameters were counted or measured: number of ramets, number of fertile and sterile fronds, mean height of ramets and total frond area (sum of the frond size).

We also measured forest canopy cover by ocular estimation (as a percentage) at the center of the plot. Canopy cover is defined as the proportion of the forest floor covered by the vertical projection of the tree crowns (Jennings *et al.*, 1999), including foliage and plant material from species rooted both within and outside the plot.

At the ramet level, a one-way ANOVA was applied in order to determine the differences in the number of fertile fronds (response variable) between ramets with different numbers of sterile fronds (categorical predictor). In order to estimate the differences between the means, the Tukey HSD post-hoc multiple comparison test for unequal samples was applied, with a significance level of 0.05. At the plot level, regression analysis was used to investigate the responses of density and frond traits (dependent variables) to the forest canopy cover gradient (independent variable), and the correlations between dependent variables were identified by a correlation matrix. All the above analyses were only performed in plots containing ramets without damage due to

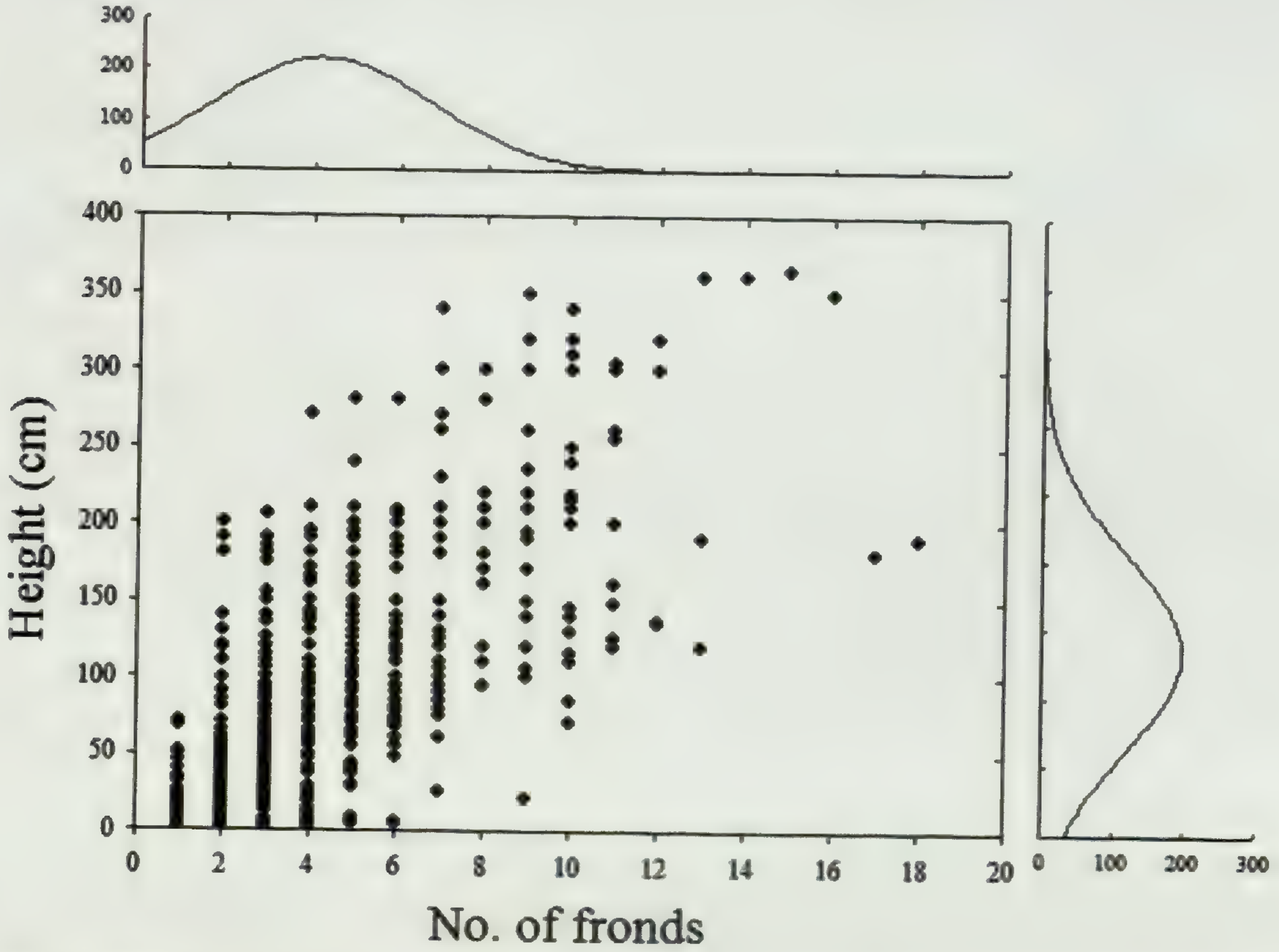

FIG. 2. Relationship between total number of fronds (sterile and fertile) and height of ramets (no. = 876). The frequency distribution of ramets is shown alongside the axes.

grazing. Ramets and fronds with mechanical breakages were excluded from all analyses. A two-way ANOVA was used to test the effects of canopy cover (divided into open and forest), grazing (divided into grazed and ungrazed), and their interaction on frond traits (dependent variables) such as the number of ramets, number of fertile and sterile fronds, total frond area and mean height per plot. In this analysis, the open category included plots with canopy cover between 0 and 50% and the forest category included plots with canopy cover between 51 and 100%. The statistical analyses were performed using STATISTICA (StatSoft Inc., 2005).

RESULTS

Density and frond traits.—A total of 1704 ramets containing 6416 fronds were investigated, of which 5643 were sterile and 773 fertile. There were 876 ungrazed ramets with 3612 fronds, of which 586 (19.4%) were fertile.

The increase in the height of ramets was correlated with an increase in the number of fronds (Fig. 2). Most of the ramets consisted of 3–5 fronds and had a height of between 100 and 150 cm. One ramet had 18 fronds and the largest ramet reached a maximum height of 365 cm.

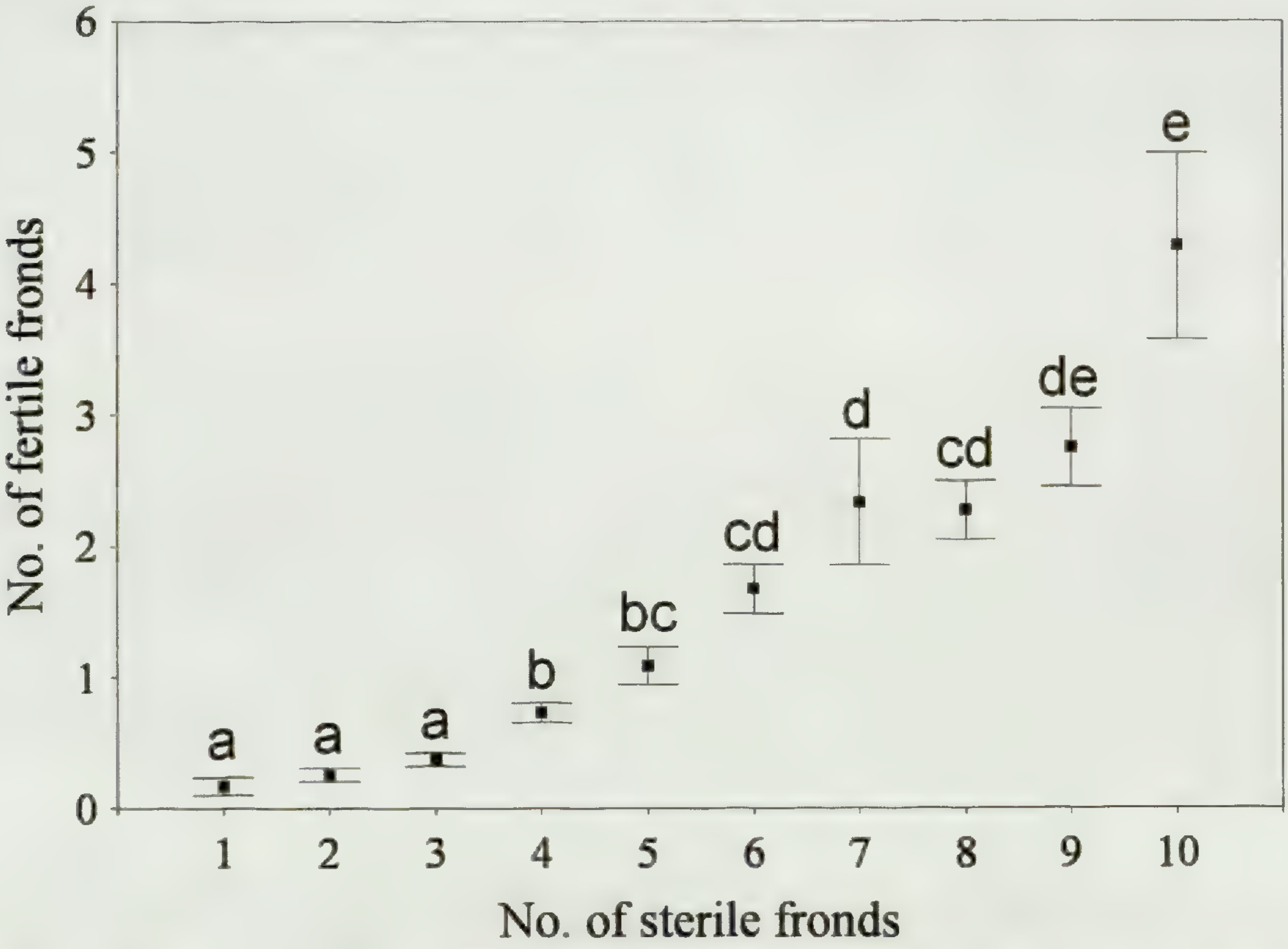

FIG. 3. Differences in number of fertile fronds between ramets (n = 876) with different numbers of sterile fronds. Symbols represent the mean (dark squares) and standard error (vertical bars). Different letters indicate significant differences according to Tukey's test for unequal samples at the $P < 0.05$ level.

There were significant differences in the number of fertile fronds between groups of ramets with different numbers of sterile fronds (one-way ANOVA, $F_{9,866} = 36.73$, $P < 0.01$). However, the number of fertile fronds also varied between groups of ramets and Tukey's test showed significant differences between groups with more than three sterile fronds (Fig. 3).

The number of fertile and sterile fronds, total frond area and mean height of ramets were all positively and significantly correlated (Table 1). However, while the mean height of ramets was strongly correlated with the number of fertile fronds and total frond area, it was weakly correlated with number of

TABLE 1. Matrix of correlation coefficients between density and frond traits sampled at plot level (no. = 78). * indicates significance at the 0.01 level.

	Ramets (no.)	Fertile fronds	Sterile fronds	Total frond area
Fertile fronds (no.)	0.282			
Sterile fronds (no.)	0.905*	0.547*		
Total frond area (m^2)	0.417*	0.785*	0.666*	
Mean height of the ramets (cm)	0.083	0.791*	0.379*	0.831*

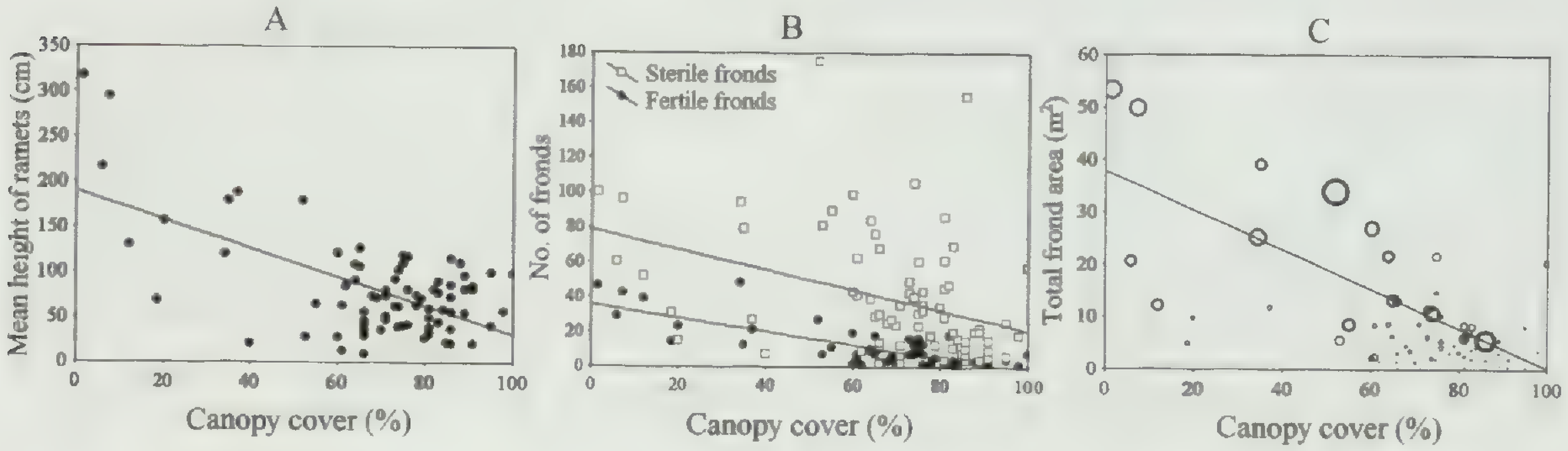

FIG. 4. The relationship between percent forest canopy cover and mean height of ramets (A), number of sterile fronds (white squares) and fertile fronds (black dots) (B), and total frond area, with circle sizes proportional to the number of fronds (C) which ranged from 1 to 202 (mean = 46.3 ± 41.9 SD). All data were collected in ungrazed plots (no. = 78).

sterile fronds. The density of ramets (no. of ramets/plot) showed no significant correlation with mean height or the number of fertile fronds.

Response to forest canopy cover gradient.—Forest canopy cover ranged from 1–100%, but most plots (57 out of 78 the ungrazed plots) had over 60% cover.

The mean height of ramets ($R^2 = 0.382$, $P < 0.01$; Fig. 4a) and number of fertile fronds ($R^2 = 0.614$; $P < 0.01$; black dots in Fig. 4b) responded negatively to increasing canopy cover. Canopy cover had a weak influence on the number of sterile fronds ($R^2 = 0.131$; $P < 0.01$; white squares in Fig. 4b), but was negatively correlated with the total frond area ($R^2 = 0.363$; $P < 0.01$) and the number of fronds ($R^2 = 0.476$; $P < 0.01$; Fig. 4c). The relationship between the number of ramets and forest canopy cover gradient was not significant.

Effect of deer grazing and interaction between grazing and canopy cover.—Fronds grazed by deer were detected in 50 plots belonging to 21 populations, while the fronds in the 78 ungrazed plots belonged to 38 populations; in 12 populations both types of plot (grazed and ungrazed) were detected. Two-way ANOVA revealed significant effects of grazing on the number of fertile fronds ($F_{1,124} = 26.01$; $P < 0.01$), mean height ($F_{1.124} = 18.88$; $P < 0.01$) and total frond area ($F_{1,124} = 10.64$; $P < 0.01$). In the grazed plots these frond traits were lower than in ungrazed plots (their mean values decreased by approximately 50%, 30% and 26% respectively, see Table. 2). In addition, interaction between canopy cover and grazing significantly affected the number of fertile fronds ($F_{1.124} = 19.20$; $P < 0.01$), mean height ($F_{1,124} = 10.18$; $P < 0.01$) and total frond

TABLE 2. Mean ± SD of number of *O. regalis* ramets and frond traits in all plots, in ungrazed plots and in plots grazed by deer.

	All plots (128)	Ungrazed plots (n = 78)	Grazed plots (n = 50)
Ramets (No.)	13.31 ± 11.31	11.23 ± 10.16	16.56 ± 12.31
Fertile fronds (No.)	6.04 ± 9.36	7.55 ± 10.87	3.68 ± 5.64
Sterile fronds (No.)	44.09 ± 40.08	38.7 ± .34.92	52.48 ± 47.74
Total frond area (m^2)	7.57 ± 9.31	8.42 ± 10.45	6.23 ± 7.05
Mean height (cm)	70.14 ± 48.67	79.86 ± 55.91	54.96 ± 28.90

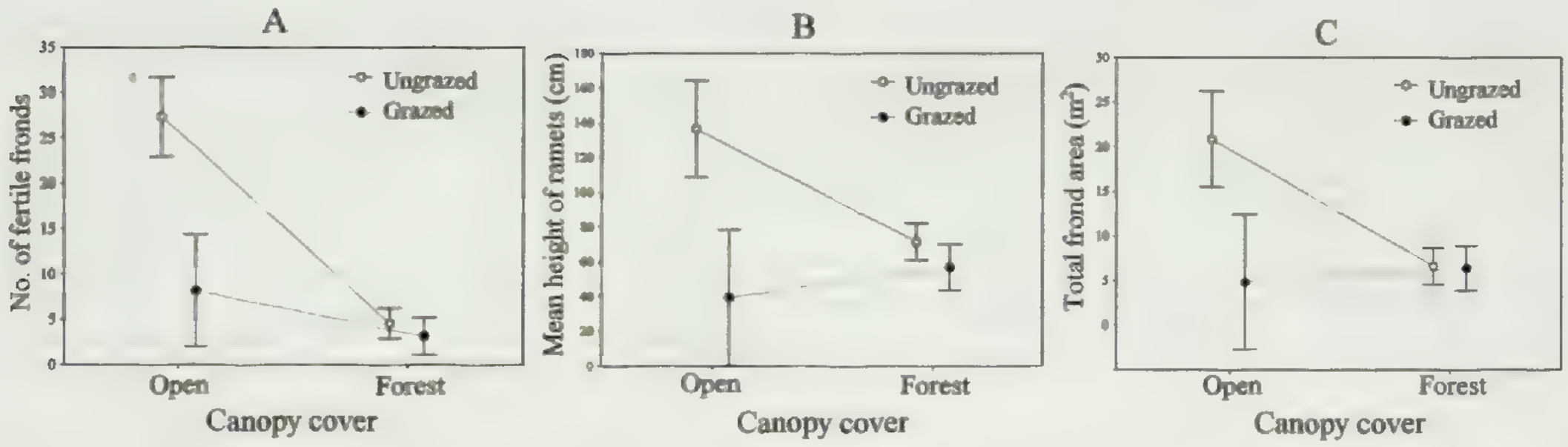

FIG. 5. Effects of grazing (grazed and ungrazed) and canopy cover (open and forest) on the number of fertile fronds (A), mean height (B) and total frond area (C). Means (dots) and 95% confidence intervals (vertical bars) are shown. The interactions were significant at 0.01 level (two-way ANOVA).

area ($F_{1,124} = 10.09$; $P < 0.01$). The effect of grazing on these frond traits was greater in open than in forest plots (Fig. 5). Grazing and the interaction between canopy cover and grazing were found to have no significant effect on the number of sterile fronds and number of ramets.

DISCUSSION

The results showed that the number of fertile fronds increased with the number of sterile fronds. However, the variation in the number of fertile fronds between ramets with different numbers of sterile fronds underlines that the production of fertile fronds also depends on other factors (e.g., light and nutrients) favoring their development.

The stronger correlation between the number of fertile fronds and the mean height of ramets suggests that fertile fronds are higher than sterile fronds. The parameter of height can be related or unrelated to light and frond density in different species of ferns (see Hill and Silander, 2001). We observed that light appears to promote fertility in *O. regalis*, as the number of fertile fronds increased considerably with greater height, while these two positively correlated characteristics decreased with forest canopy cover. The analysis of total frond area and canopy cover revealed that fronds are fewer and smaller in plots under high tree cover. Odland *et al.* (2006) indicated similar results in the dimorphic fern *Matteuccia struthiopteris*. Thus, *O. regalis* performs best in conditions of full sunlight but can persist and recruit young sporophytes under a very dense tree canopy.

Until the early 1900s this fern was reported in many locations in central Italy, prevalently in marsh and lake environments. After 1950 these environments underwent considerable land use changes for both intensive farming and industry, due to the increasing human population (Falcucci *et al.*, 2007). These changes led to the extinction of many *O. regalis* populations and the remaining populations are mainly located in streams and springs within forests. For these reasons we collected much more data under high tree cover than in open habitats. Within forests, the sunny habitats preferred by *O. regalis*

may be found in both human-altered environments (alongside paths) and in natural gaps (i.e., spring swamps) (Landi and Angiolini, 2008). However, as moist and open habitats are appropriate sites for the growth and production of fertile fronds, conservation efforts should focus primarily on the maintenance of marshes in open habitats and spring swamps in forest habitats.

On the Isle of Rum, Wood (2000) notes that *O. regalis* is very sensitive to grazing and is present in the few places that are inaccessible to deer, while it is a common component of the vegetation on other ungrazed islands nearby. In Tuscany, deer have been observed grazing *O. regalis* fronds and bark stripping has been detected on the trees in the surrounding vegetation that is more susceptible to this damage (i.e., *Alnus glutinosa, Frangula alnus, Populus tremula, Carpinus, Fraxinus* and *Quercus*) (see Gill, 1992).

The results presented here show that the number of fertile fronds, the mean height of ramets and total frond area is significantly lower in grazed plots than in ungrazed plots. The interaction between grazing and canopy cover indicates that grazing affects more open than forest sites. The number of ramets and sterile fronds were unaffected by grazing. Moreover, the number of ramets was higher in grazed plots than ungrazed plots. This may be related to the higher abundance of ramets in open areas (i.e., spring swamps, see Landi and Angiolini, 2008), which are more affected by grazing.

Our data suggest that fertile and sterile fronds of *O. regalis* respond differently to the availability of light and to grazing. These results can be used to formulate the following alternative explanations: i) deer prefer fertile fronds; ii) ramets with damaged fronds become weakened and produce fewer fertile fronds after grazing by deer. Ferns can be a valuable source of food for deer in winter, before leaves have appeared on woody vegetation (Healy, 1971). However, this cannot be the case with *O. regalis* as it is a deciduous herbaceous fern with fronds that develop in May and become senescent in September. Thus, this fern can only be grazed in summer, when food is fully available in the surrounding vegetation.

ACKNOWLEDGEMENTS

The authors thank Barbara Anselmi (Faunal Resources and Nature Reserve Service, Siena) for her contribution during field data collection and two anonymous referees.

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American Fern Journal 100(3):148–158 (2010)

Effects of Harvesting on Population Structure of Leatherleaf Fern (*Rumohra adiantiformis* (G. Forst.) Ching) in Brazilian Atlantic Rainforest

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Abstract.—Among ferns, the leatherleaf fern (*Rumohra adiantiformis*) is of particular economic importance. The fronds are sold around the world for flower arrangements. In Brazil, these fern fronds represent an income source for numerous households, working with a traditional management system with a maximum of three annual collections in the same area. The purpose of this study was to evaluate the demographic structure of managed *R. adiantiformis* populations in the south of Brazil and verify the sustainability of the harvesting activity in this region. The study was conducted in an area of Atlantic Rainforest in the State of Rio Grande do Sul. The demographic structure of the managed populations was evaluated in permanent plots, where fronds in the different development phases were counted quarterly for one year. Rainfall was identified as the main factor influencing bud emergence and development. A reduction in *R. adiantiformis* populations related to forest regeneration was also observed. Fast frond regeneration and the absence of differences between managed and non-managed populations concerning the proportions of buds and young fronds corroborate the ecological sustainability of the management system used by the local harvesters. The results obtained differ largely from those of populations studied in South Africa, where *R. adiantiformis* plants seem unable to maintain frond density and size when harvested. These contrasting results are probably related to climatic differences between these areas, especially annual rainfall, which is higher in Brazil allowing for the rapid regeneration and sustainable management of this resource in the Brazilian Atlantic Rainforest.

Key Words.—sustainable management, tropical forests, demography, non-timber forest products

Non-timber forest products (NTFPs) are biological non-wood resources/products, which can be obtained from natural and managed forests for consumption and/or trade (Peters, 1994). NTFPs are a source of social and economic benefits, particularly for local or traditional communities. More adequate management regimes for the exploitation of a great number of NTFPs should, however, be determined (Shanley *et al.*, 2002). Information about the autoecology and management effects on populations is available only for a limited number of species.

Despite the economic importance of several fern species around the world, there is still surprisingly little information on the impacts of frond harvesting on fern populations (Ticktin *et al.*, 2007). One of the most important NTFPs in Brazil is the pteridophyte *Rumohra adiantiformis* (G. Forst.) Ching (leatherleaf fern). This species is of particular economic importance among ferns and its

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TABLE 1. Geographical placement and status of leatherleaf fern (*Rumohra adiantiformis* (G. Forst.) Ching) study populations.

Population	Geographic coordinates	Altitude (m)	Status	Harvest period
1	29°55′S/50°23′W	253.91	Managed	Sep 2004
2	29°64′S/50°14′W	355.09	Managed	Aug 2004; Aug 2005
3	29°60′S/50°15′W	225.07	Managed	Nov 2004; Mar 2005; Jun 2005
4	29°56′S/50°23′W	245.02	Non-managed	Zero

fronds are sold all over the world for floral arrangements. Leatherleaf fern fronds are particularly popular thanks to their long post-harvest life.

In Brazil, the fronds are extracted mainly from the natural environment and harvested in Atlantic Rain Forest areas in the south and southeast of the country (Conte *et al.*, 2000; Coelho de Souza, 2003). More than 50% of the *R. adiantiformis* traded in Brazil comes from Rio Grande do Sul (Anama, 2002) and the major source of income of about 2000 rural households in the northeastern region of Rio Grande do Sul is based on harvesting and trading *R. adiantiformis* fronds (Coelho de Souza *et al.*, 2006).

Although the plants are not collected (only the fronds), frond harvesting may cause impacts on *R. adiantiformis* populations by reducing rhizome growth, frond density and size, and effective population size (Milton and Moll, 1988; Geldenhuys and Van der Merwe, 1988; Baldauf *et al.*, 2008). Detailed knowledge about the impacts of the management system used by local harvesters is therefore required to determine conditions of sustainable extraction of this resource. The purpose of this study was to evaluate the demographic structure of managed populations of *R. adiantiformis* in the south of Brazil, as well as to verify the sustainability of the harvesting activity in this region.

MATERIAL AND METHODS

Study area and sampled populations.—Four populations were studied in Maquiné, a municipality in the northern coastal region of the State of Rio Grande do Sul, Brazil (Table 1). This area is considered the southern limit of the distribution of Atlantic Rainforest in Brazil. Populations one, two, and three were managed according to traditional knowledge, in which harvesting occurs in any month of the year, with a maximum of three annual collections permitted in the same area. In general, all fronds longer than 30 cm are harvested.

Population four was evaluated in an area of species occurrence where fronds are not harvested; this population served as a reference of the population demographic structure without exploitation, which is rare in the study area. This population is located in a private area with the same ecological

TABLE 2. Correlation coefficient of the demographic and phytossociological descriptors for the first three ordination axes of the principal component analysis (PCA). The values in bold were used for interpretation of the axis. The first three axes explained 76.8% of the total data variation.

Descriptors	Axis 1	Axis 2	Axis 3
No. of buds (BUD)	0.66	−0.07	0.13
No. of young fronds (YOU)	**0.74**	0.39	0.28
No. of adult fronds (ADU)	**0.82**	0.15	0.32
No. of dead fronds (DEA)	0.67	−0.17	−0.49
Total no. of fronds (TOT)	**0.97**	0.12	0.10
No. of fertile fronds (FER)	0.47	−0.53	−0.55
Basal area (BA)	**−0.78**	0.22	−0.01
Diameter at Breast Height (DBH)	−0.28	**0.82**	−0.29
No. of trees (TRE)	−0.40	**−0.70**	0.43

conditions as the others, but its owner decided to allow forest regeneration to take place and maintain the area for biological conservation. It was impossible to conduct a replication of the unharvested area due to the lack of populations large enough for experimental studies and free from exploitation.

Evaluation of demographic structure.—Demographic structure was characterized using four 5 × 5 m plots in each of the four study populations. Since *R. adiantiformis* is a rhizomatous plant, the fronds were considered individual units. The fronds were counted at each development stage, in four categories: buds, young fronds, adult fronds, and dead fronds. The presence of fertile fronds was verified as well. The fronds were counted quarterly for one year, with evaluations in August 2004, November 2004, February 2005, May 2005 and August 2005. In those cases in which harvesting and monitoring occurred in the same month, the evaluations were carried out before frond harvesting.

The respective environments of the sampled units were characterized based on data of the existing tree and shrub plants in the plots. The parameters evaluated were: number of plants (height of over 1.30m, independent of the species) and diameter at breast height (DBH – 1.30m), the latter measured with a pachymeter. This evaluation was carried out in an area of 8 × 8 m in the center of the plots described above and all non-fern species were measured.

Data analysis.—Correlation analysis and principal component analysis (PCA) were used for the demographic data analysis and possible relations with phytossociological descriptors. The demographic descriptors were: BUD (number of buds), YOU (number of young fronds), ADU (number of adult fronds), DEA (number of dead fronds), TOT (number of total fronds) and FER (number of fertile fronds). The phytossociological descriptors were: BA (basal area), DBH (diameter at breast height) and TRE (number of trees). The descriptors that contributed the most to axis formation, with correlations equal to or higher than 0.70, were used in the interpretation. The population structure was compared using the Kolmogorov-Smirnov test with Bonferroni correction for multiple comparisons. Statistical analyses were performed using Statistica 6.0 (StatSoft, 1998) and MVSP (Multivariate Statistical Package) version 3.12 d (Kovach, 2001).

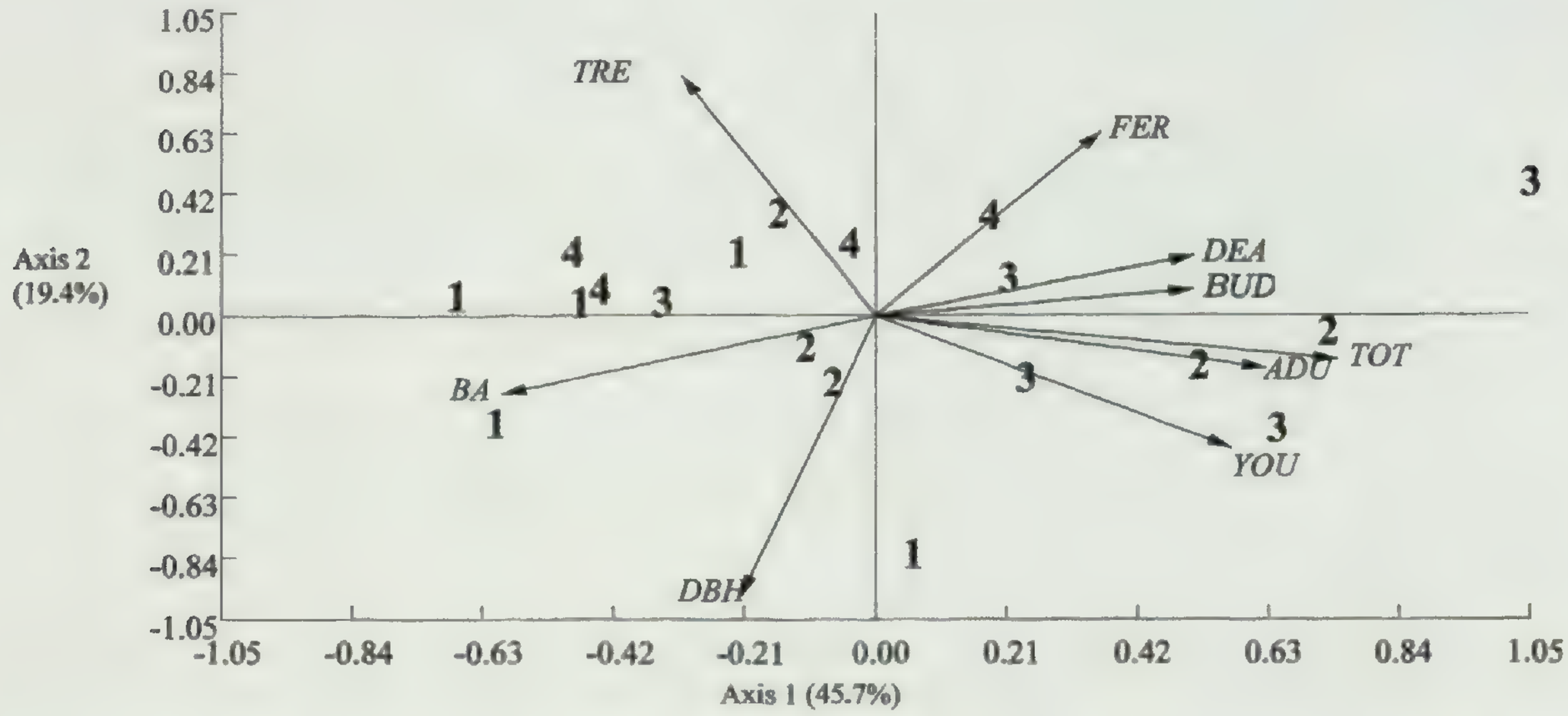

FIG. 1. Principal component analyses of phytossociological and demographic data of four *Rumohra adiantiformis* populations. The numbers represent the sample units (plots) of each population and the descriptors are: BUD (number of buds), YOU (number of young fronds), ADU (number of adult fronds), DEA (number of dead fronds), TOT (number of total fronds), FER (number of fertile fronds), BA (basal area), DBH (diameter at breast height), and TRE (number of trees).

RESULTS

Environmental aspects.—Large variations in the number of fertile fronds, as well as in the number of fronds in each development category, were observed in the populations studied. The number of fertile fronds was the most variable. The coefficients of variation obtained for each category were: buds (75.1%), young fronds, (51.9%) adult fronds, (47.7%), dead fronds (39.3%) and fertile fronds (144.8%). The existence of seasonality was verified in the formation of new fronds (buds). Bud emergence was more intensive at the end of winter and the beginning of spring (between August and September). A correlation between some climatic factors and frond production in the species was also observed. The correlation between the number of buds and mean monthly rainfall was negative ($r^2 = -0.58$; $p < 0.01$) and positive between the number of buds and rainfall in the three months before evaluation ($r^2 = 0.67$; $p < 0.01$). A negative correlation between the basal area and the average number of fertile fronds throughout the studied period was also verified ($r^2 = -0.43$; $p < 0.01$).

The first three axes of the Principal Components Analysis explained 76.8% of the variation in the data. The first axis (45.7%) showed a correlation between the *R. adiantiformis* population structure and the forest succession stage (Fig. 1). The correlations of the descriptors YOU (number of young fronds), ADU (number of adult fronds) and TOT (number of total fronds) with the main component axis one were high and positive (Table 2). On the other hand, the descriptor BA (basal area) was high and the correlation with axis one, negative. Consequently, the number of young, adult and total fronds is higher in environments of lower basal area of tree and shrub species, and vice-versa.

The second and third axes explained 19.4% and 11.7% of variance, respectively, but the only descriptors with values equal to or higher than 0.70 were DBH (Diameter at Breast Height) and TRE (No. of trees) in axis two. Thus, no associations between demographic descriptors and phytossociological parameters shown in axis two and three were above the value considered for interpretation (0.70).

Harvesting potential and monitoring indicators of R. adiantiformis *populations.*—The patterns of population one and two were similar in terms of number and proportion of each development phase during the year (Fig. 2). The number of adult fronds dropped with the first collection, but in the following evaluations the values were similar to those prior to frond harvesting. Likewise, in population three, the recovery in frond number and proportion after harvesting was fast. Bud production was intensive in August 2005, in all sampled populations (including the non-harvested area).

In general, frond regeneration after the cuts was rather fast in the managed populations, evidenced by the adult frond numbers found after the collections (Fig. 2). It was also noticed that the demographic dynamism of natural *R. adiantiformis* populations is considerable, as evidenced by the variations in frond number and proportion over the study year in different development phases of the non-managed population (population four) (Fig. 2). However, between August 2004 and August 2005, the relative frequency of adult fronds decreased in this population.

No significant differences were observed among the traditional management systems (populations one, two and three) in relation to the proportion of frond categories (Table 3). However, populations one and two revealed differences as compared to population four (non-managed), due to distinct proportions of adult and dead fronds. In population two, the number of adult fronds was higher than in the non-managed population (population four). Concerning the number of dead fronds, values were higher in population four (non-managed), differing from populations one and two. The proportion of fertile fronds was lower in population one when compared to population four. The other comparisons for this trait indicated no differences.

Discussion

Environmental aspects.—The high variation found in the initial frond development phases has been reported in other studies on this species (Milton, 1987; Geldenhuys and Van der Merwe, 1994; Conte *et al.*, 2000), and may reflect the remarkable seasonality of bud emersion in the species. According to Geldenhuys and Van der Merwe (1988), bud production begins between May and July in South Africa and reaches a peak between September and November. In the South of Brazil, the most intensive period of bud production is in August and September (Anama, 2002; Baldauf, 2006). Intensive bud production in these months was also confirmed in the first year of this study. This occurred in both harvested and non-harvested areas suggesting that this emergence is not related to the management regime employed (Fig. 2).

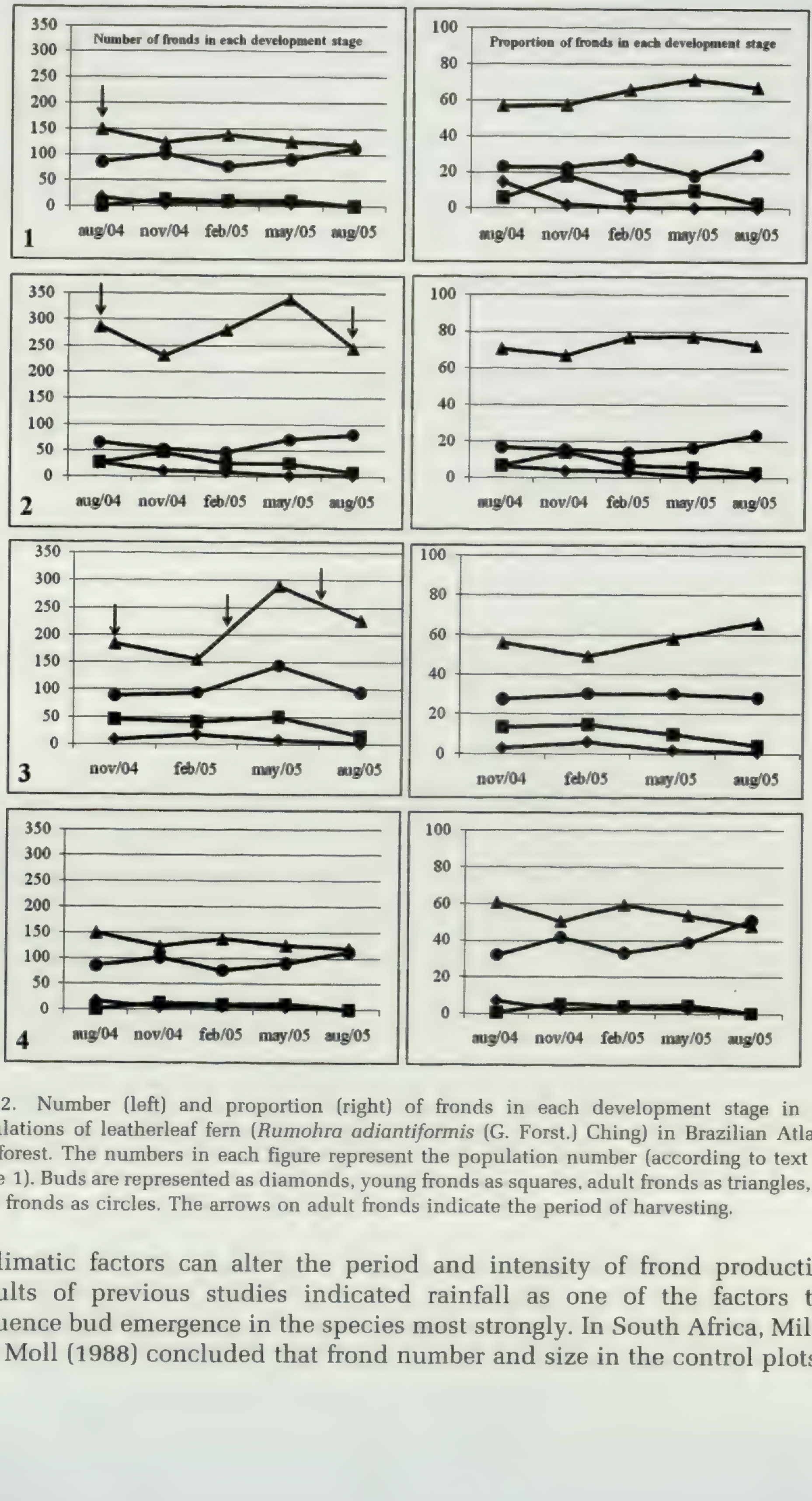

FIG. 2. Number (left) and proportion (right) of fronds in each development stage in four populations of leatherleaf fern (*Rumohra adiantiformis* (G. Forst.) Ching) in Brazilian Atlantic Rainforest. The numbers in each figure represent the population number (according to text and Table 1). Buds are represented as diamonds, young fronds as squares, adult fronds as triangles, and dead fronds as circles. The arrows on adult fronds indicate the period of harvesting.

Climatic factors can alter the period and intensity of frond production. Results of previous studies indicated rainfall as one of the factors that influence bud emergence in the species most strongly. In South Africa, Milton and Moll (1988) concluded that frond number and size in the control plots of

TABLE 3. Values of Kolmogorov-Smirnov test (D_{max}) with Bonferroni correction (α_B) (above the diagonal) and p-values (below the diagonal) for proportions of fronds in each development stage. Values followed by an asterisk differ significantly considering α_B. The last column shows Kruskal-Wallis test for the proportion of fertile fronds. Values followed by the same letter do not differ from each other according to the Dunn test (column comparison).

Population	1	2	3	4	Fertile Fr.
1	X	0.07	0.05	0.16*	1.6a
2	>0.05	X	0.12	0.23*	4.1ab
3	>0.05	<0.05	X	0.12	4.8ab
4 (control)	<0.01	<0.01	<0.05	X	19.3ab

their experiments were positively associated with rainfall in the months prior to evaluation. Conte *et al.* (2000) also stated that in the spring of 1999 the production of new fronds in the Southwest of Brazil decreased remarkably, due to low soil moisture, when rainfall was less than half of the long-term averages for the same period in previous years. Ticktin *et al.* (2007) noted that changes in frond density and rates of frond production over time coincide closely with changes in precipitation levels over time and that seasonal rainfall rates were significantly correlated with rates of frond production in two Hawaiian fern species. Thus, the lower number and proportion of buds found in the evaluation carried out in August 2005 (when compared with 2004) are probably due to the average rainfall value of the previous three months, which was approximately half the value measured in 2004.

In relation to the vegetative propagation of the species, water availability in the months prior to bud formation possibly results in water reserves for the plant, which reduces the chances of bud desiccation after emergence. On the other hand, Stamps *et al.* (1994) claimed that the emergence of new fronds is associated with soil temperature, which increases during the sunny periods; this explains the inverse and significant correlation between monthly precipitation and frond production observed here.

Our results further show a probable reduction in *R. adiantiformis* populations caused by forest regeneration, as shown in the principal component analysis. This analysis showed that when the basal area of a particular location increases, the number of young and adult fronds tends to decrease. This tendency was also confirmed by Milton and Moll (1988), who observed that the density of *R. adiantiformis* populations in South Africa was negatively correlated with tree height and canopy cover. Local harvesters mentioned this aspect as a critical limit for leatherfern harvesting in the Atlantic Rainforest (Baldauf *et al.*, 2008). Additionally, the lower number of fertile fronds registered for population one is probably related to the higher basal area of the plots where this population occurs. The results presented here show that fertile fronds are more common in areas with more solar radiation. This fact emphasizes the need for open canopy areas to maintain viable *R. adiantiformis* populations.

Harvesting potential and monitoring indicators for R. adiantiformis.—One of the most remarkable tendencies of *R. adiantiformis*, and highly relevant for species management, is the rapid frond regeneration after harvesting. In general, frond regrowth of populations is fast, as evidenced by the number and proportion of adult fronds in the evaluation carried out after the collection. The results for population three are particularly noteworthy, since they represent the most commonly used harvesting frequency in the region under study (Baldauf *et al.*, 2007).

The results obtained here differ largely from those of populations studied in South Africa, where *R. adiantiformis* plants seem unable to maintain frond density and size when harvested (Milton, 1987; Milton and Moll, 1988; Geldenhuys and Van der Merwe, 1988). According to Milton (1991), a period of five years is insufficient for *R. adiantiformis*, under natural conditions in the indigenous forests of the southern Cape, to recover from total or partial defoliation. In these forests, only 50% of all pickable fronds per plant may be harvested and the harvesting cycle was extended to 15 months to give plants sufficient time to recover (Geldenhuys and Van der Merwe, 1994). These contrasting results may be related to climatic differences between the areas. According to Stamps *et al.* (1994), *R. adiantiformis* grows faster under a high-temperature regime than under a low-temperature regime. In another study (Strandberg, 2003), it was observed that the production of new fronds and the time required for leaves to mature were also strongly associated with degree days, daylight hours, solar radiation, and soil temperature. However, there are no remarkable differences in the temperature regime of the areas in question. Thus, other factors might be involved.

One possibility, already mentioned, is the influence of rainfall on the production of fronds. Annual rainfall in the Maquiné area varies from 1400 mm to 1800 mm (Hasenack and Ferraro, 1989), whereas the annual rainfall in the South African area ranges from 920 mm to 1250 mm (Milton and Moll, 1988). Since the emergence of new fronds shows a positive correlation with rainfall, this could be an explanation for the differences found.

Milton (1991) attributes the slow recovery in South African populations in part to long leaf longevity in this species. In Brazilian natural populations, fronds are also very long-lived (Anama, 2002; Conte *et al.* 2000), but despite this fact they can recover rapidly. Additionally, the higher harvest rates applied to Brazilian populations as compared with the rates employed in South Africa could cause reductions in frond production, as reported by Milton (1991), but such reductions do not occur in Brazil (at least not when the traditional management system is employed). Geldenhuys and Van der Merwe (1988) point out that frond production in South Africa appears to be nutrient limited, and frond removal also reduces nutrient availability. Therefore, another possibility is that, in Brazilian populations, there would be no nutrient limitation to the species growth. Nevertheless, further studies are necessary to confirm the influences of edaphoclimatic conditions on the population structure of *R. adiantiformis*.

Conte *et al.* (2000) suggested that a cutting cycle of five events each year (cycles of two months during the rainy season and of three months in the dry season) could maintain the sustainability of *R. adiantiformis* populations in the State of São Paulo, in the southeast region of Brazil. However, in an experimental study conducted in Rio Grande do Sul, in the south of Brazil, Anama (2002) applied different harvesting frequencies and concluded that traditional harvesting, which consists of a maximum of three annual cuts, was the only treatment that would not affect frond number and size.

Differences in ideal harvesting frequencies between the South and Southeast regions of Brazil reinforce the influence of rainfall on frond production, since the annual rainfall in the area where Conte *et al.* (2000) conducted their study is 2300 mm (CEPAGRI, 2010), higher than the values observed in other areas where the species is harvested in Brazil. These differences in appropriate harvesting regimes also show that monitoring indicators must be determined on a regional scale.

The results reported here agree with those obtained in the study by Anama (2002), since they reinforce the absence of negative impacts of traditional harvesting on the species population structure. The differences in dead frond proportions found among populations one and two and population four (control) probably reflect the fact that the harvesters are removing adult fronds from populations one and two. Thus, less dead fronds would be expected in these populations than in the control, where fronds die naturally or remain on the plant for a long time. Nevertheless, no differences were observed on the proportions of buds and young fronds among the populations under investigation, which may be considered a sign of harvesting sustainability. According to Peters (1996), the first sign of over-exploration of a plant population is expressed in its size class distribution. In most species, the most visible effect of over-exploration is a reduction in the number of seedlings and young plants. Since *R. adiantiformis* is a rhizomatous species, the initial frond development phases (buds and young fronds) are understood as equivalent to seedlings and young plants of tree species in order to monitor the harvesting activity.

In view of the fact that in managed populations the variation in the proportion of young fronds is lower than of buds, the former was considered a better indicator of the demographic dynamism, characteristic of the species and of possible impacts of frond harvesting. The reference values for this indicator are variable, due to a period of intensive frond formation at the end of the winter (August and September). Based on the fact that there is no difference in the proportion of young fronds in the managed populations when compared with the non-managed population, references values were established for monitoring. These values comprise the range of proportions obtained in all studied populations plus a safety margin. Therefore, the value of young fronds should be between 10% and 20% in the period between September and December (after bud formation), whereas in other periods of the year a proportion between 5% and 15% would be expected.

The demographic structure of leatherleaf fern is largely influenced by climatic factors and forest succession and must therefore be continuously monitored over several years. It is also important to consider that the results and monitoring indicators presented here are based on studies carried out at few sites and should be revised periodically. In cases that require a more in-depth analysis, it is possible to analyze the genetic diversity of the managed populations, based on allozyme markers. By this technique, the main genetic diversity indexes can be determined; in case of population overexploitation, these values may be reduced (Baldauf *et al.*, 2008). Once the demographic data are relatively easy to obtain, evaluations may include the local harvesters of the collection areas, helping to evaluate the populations in a participatory monitoring process.

Finally, it should be emphasized that the low ecological impact of the traditional management systems is not sufficient for the maintenance of a leatherleaf fern population, since it is a pioneer species that tends to disappear during forest succession. Hill and Silander Jr. (2001) studied the pteridophyte species *Dennstaedtia punctilobula* (Michx.) T. Moore and understood that any event that maintains or results in canopy openness (tree death caused by disease, windthrow or forest harvesting, or the elimination of a shrub layer by browsing) favors the persistence of *D. punctilobula*. Analogously, the maintenance of *R. adiantiformis* populations also depends on further management measures that would improve productivity, as for example the management of "capoeira" (naturally regenerated second-growth forest) areas. This, along with the farming of perennial agricultural species, could be a way to guarantee income and food security to the harvesting families.

ACKNOWLEDGEMENTS

The authors are indebted to the harvesters for their collaboration and for sharing their knowledge; to the land owners for granting access to the study area; to Núcleo de Pesquisas em Florestas Tropicais (NPFT-UFSC) and NGO Anama (Ação Nascente Maquiné) for support in field work; and to Coordenação e Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support. Thanks also to Dr. Alexandre Mariot for providing the articles on *Rumohra*, and to Dr. Flavio A. M. dos Santos for suggestions on statistics.

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American Fern Journal 100(3):159–166 (2010)

Recovery of Viable Germplasm from Herbarium Specimens of *Osmunda regalis* L.

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ABSTRACT.—The conservation of pteridophytes presents a demanding challenge because many species in the world are thought to be threatened with extinction, as they are very sensitive to environmental disturbance. *Ex situ* actions provide an important conservation strategy, so the Germplasm Bank of Tuscia Botanic Garden, with the Herbarium UTV (Tuscia University, Viterbo–Italy), has undertaken a project for the conservation of threatened pteridophytes of the Italian flora, like *Osmunda regalis*, the Royal Fern, a species with chlorophyllous spores that is declining in Italy because it is linked to vulnerable habitats. As a part of the project, this work presents first results of *in vitro* reproduction of the Royal Fern using spores from *exsiccata* (UTV samples collected and dried in 1989 and 2001). Our results also highlight the value of herbarium specimens in biodiversity conservation, providing a useful method to reproduce species that are threatened or extinct in the wild, at least at a local level, so as to plan eventual reintroductions using the native germplasm.

KEY WORDS.—*Osmunda regalis*, chlorophyllous spores, herbarium, *in vitro* reproduction, biodiversity conservation

The conservation of pteridophytes presents a demanding challenge, but this highly diverse group is underrepresented on the global Red List. The International Union for Conservation of Nature (IUCN) listed 770 species of pteridophytes world-wide as threatened (Walter and Gillett, 1998), but only 211 in the 2008 Red List (http://www.iucnredlist.org) due to difficulty in assessing species with the new criteria because of significant gaps in the knowledge of their biology, distribution and conservation status (Chandra *et al.*, 2008). Although the species evaluated by IUCN only represent 1.6% of the global described species (13,025) (Arcand and Ranker, 2008), over 10% of the species in the world are thought to be threatened with extinction (Jermy, 1990) and many others are declining as they are very sensitive to environmental perturbations. Moreover, a tendency to be concentrated in endangered regions and habitats, and frequent local endemism, especially on islands, means that a significant proportion of species may be at risk (Given, 2002).

Successful *in situ* conservation of ferns and their-allies within their natural habitats should always be the primary goal of their conservation. However, when this is not possible, additional methods exist to supplement and complement the *in situ* actions, and they are needed to increase the chances of survival of individual species (Page *et al.*, 1992). *Ex situ* approaches provide an

important conservation strategy (Pence, 2002; 2008), but methodologies for the long-term preservation in germplasm banks are not well established.

Pteridophyte spore viability depends on spore type, chlorophyllous and non-chlorophyllous, or taxonomic group (Lloyd and Klekowski, 1970). Biochemical and metabolic factors, depletion of respiratory substrate, loss of membrane integrity, inactivation of enzymes and growth promoters, and chromosomal aberrations or other genetic mutations are cited as possible causes of the rapid loss of viability during storage of non-green spores (Page *et al.*, 1992; Beri and Bir, 1993). In green spores, the high respiratory rate or the failure to recover photosynthetic activity after desiccation are suggested to cause the rapid loss of viability (Jones and Hook, 1970; Lloyd and Klekowski, 1970). There is a narrow window of water contents appropriate for storage at conventional temperatures, so Ballesteros and Walters (2007a, b) have proposed that damage by desiccation or cooling can be prevented by precise control of the water content within spores.

Lloyd and Klekowski (1970) have made comparisons between the viability of chlorophyllous and non-chlorophyllous spores of several species of ferns; they observed that spores with chloroplasts germinate more rapidly than spores without chloroplasts (within one or two days after sowing), but this ability to germinate declines rapidly with their age. Moreover their results show that, under laboratory conditions (room temperature), green spores die early, averaging a life-span of only 48 days, while non-green spores live on average up to 2.8 years. Lebkuecher (1997) suggested that the extremely short viability of chlorophyllous spores of *Equisetum hyemale* L. may result from the inability to recover the photosynthetic competence when the spores were re-wetted after desiccation. Usually, the viability of chlorophyllous spores can be measured in days and weeks rather than in months and years (Lloyd and Klekowski, 1970).

Factors affecting viability of spores (green and non-green) are rather clear, but little is known about the factors affecting spore longevity under herbarium conditions. There are several reports of viability of seeds after lengthy periods of storage in herbaria (Freeman, 1983; Hill, 1983; Willan, 1987; Bowles *et al.*, 1993; Moreno Casasola, 1996), but there are only a few studies concerning pteridophytes. These reports indicate that the non-chlorophyllous spores from herbarium collections of some fern species remain viable for decades. Jonhson (1985) reported that the spores of certain *Marsilea* species were capable of germination after 99–100 years and Windham *et al.* (1986) provided germination data of 50-years-old spores of *Pellaea truncata* Goodding. This kind of longevity is quite common for species with non-chlorophyllous spores that live in xeric habitats, while mesophilic species, such as *Cystopteris protrusa* (Weath.) Blasdell e *Woodsia obtusa* (Spr.) Torrey, lose the capability to germinate after a few years (Windham and Haufler, 1986).

For herbarium specimens, the most important factor controlling spore longevity, aside from the biologic features of the ferns, seems to be the type of treatment used to reduce insect infestations. Viability declines rapidly in herbarium specimens treated with chemical methods and heat, while exposure

to low temperatures seems to have few negative effects on species with non-chlorophyllous spores (Windham and Haufler, 1986).

The Germplasm Bank of the Botanic Garden of Tuscia with the Herbarium UTV (Tuscia University, Viterbo - Italy) has undertaken a project for *ex situ* conservation of threatened pteridophytes of the Italian flora, in order to collect and preserve spores both from natural populations and from *exsiccata* (Magrini *et al.*, 2006). As part of this project, the present work aims to reproduce *Osmunda regalis* L. *in vitro* starting from spores taken from Herbarium UTV *exsiccata*. The distribution area of the Royal Fern includes the warm and temperate regions of Europe, Asia, Africa, and America; in Italy is fairly common in Tuscany and Sardinia, while it is very rare or threatened in most Italian administrative regions (Conti *et al.*, 1997), and no longer found in four Italian regions (Conti *et al.*, 2005). The choice of this subcosmopolitan species with chlorophyllous spores derives from the observation of its increasing decline in Italy, especially considering the fragmentation and isolation of its populations in Central Italy (Landi and Angiolini, 2007). We can assume that its survival is strongly dependent upon the availability of suitable environmental conditions in vulnerable habitats such as bogs, marshes, swamps, streams, and moist forests. Therefore, increasing *ex situ* conservation strategies for the Royal Fern, like cultivation in botanic gardens and long-term storage of spores in germplasm banks, is needed for eventual re-introductions in those sites where it is already disappeared or it is threatened. Since we are not aware of published data on reproduction from *exsiccata* of species with chlorophyllous spores, we present here the first results of our study aimed to reproduce *Osmunda regalis in vitro* from spores taken from Herbarium UTV specimens, and show the recovery of viable green spores from *exsiccata* after long periods of conservation.

Materials and Methods

Among the material of the Herbarium UTV, two *exsiccata* collected from two localities within the National Park of the Tuscan Archipelago were selected. Spores of *Osmunda regalis* were collected from the *exsiccata* UTV 15510 of 05/24/1989 (17 years of storage) from Giglio Island (Grosseto - Tuscany), and UTV 16791 of 05/17/2001 (five years of storage) from M. Capanne, Elba Island (Livorno - Tuscany). The spores for the control tests were collected from a wild population (summer 2006) in a gorge near a ferruginous water stream within the Monterano Regional Nature Reserve (Rome – Latium), and they did not undergo any treatment, nor dehydration or freezing.

In UTV all incoming *exsiccata* underwent two disinfestation treatments, each a month apart: at −35°C for 3 days, thawed for 24 hours and frozen again for 3 days (4 cycles of freezing and thawing). Then, the herbarium specimens were stored in closed polyethylene bags with an internal relative humidity equal to 52% ± 2.

All spores were measured using an ocular micrometer to test their maturity, and were then soaked in distilled water for 24 h. After, they were rinsed three

times with sterile distilled water and centrifuged at 6000 rpm for 3 min between rinses (Fernandez *et al.*, 1997; 1999; Fernandez and Revilla, 2003). The spores (1,000 collected from UTV 15510, and 2,000 from UTV 16791) were sown in sterile plastic Petri dishes (6 cm diameter) containing 10 ml of Knop medium (Knop, 1865) with a Nystatin solution (100 U/ml^{-1}) added as a fungicide (Quintanilla *et al.*, 2002). The medium was adjusted to pH 5.8, and supplemented with 0.7% agar (plant tissue culture grade). The dishes were sealed with Parafilm to reduce contamination and to prevent excessive water loss (Morini, 2000). The cultures were maintained under cool-white fluorescent illumination (Osram Dulux L 36W/840 Lumilux, 2900 lm), a 12-h photoperiod (Dyer, 1979; Windham *et al.*, 1986), and a temperature of 20 ± 2°C (Dyer, 1979; Sheffield *et al.*, 2001; Quintanilla *et al.*, 2002). The dishes were examined microscopically for spore germination (defined as the first emergence of the rhizoid) and morphological studies. After germination, young gametophytes were transferred into the same medium freshly prepared, and sterile distilled water was added to enhance fertilization (Raghavan, 1989); mature gametophytes with the first sporophytes were then transferred into sterilized soil.

Results

Compared to the control germination percentage (about 100%), germination of spores collected from *exsiccata* was low (<1% for UTV 15510, 2% for UTV 16791). Moreover, we observed that spores from *exsiccata* have a longer time to germination when compared to the control, as shown in Figure 1: spores of 2001 germinated in 20 days from sowing, spores from samples of 1989 required over 30 days, whereas fresh spores germinated after 2–4 days. For the spores of 2001, we observed the subsequent phases of the reproductive cycle, until the development of the sporophytes (Fig. 2), while the germinated spores of 1989 died shortly after germination. Despite the long germination times, the development times of the gametophyte and of the sporophyte did not show significant differences compared to the control; we observed the cordate gametophytes after 35–40 days from germination, and the sexually mature ones after 60–70 days from germination. Microscopic observations (stereomicroscope and SEM) made it possible to verify the integrity and the proper development of the prothallia, and the presence of archegonia and antheridia. The first sporophytes were observed after 200–210 days after germination, but the development of the others was distributed over the next 35–60 days (Fig. 2).

Discussion

We report the first results of *in vitro* reproduction of *Osmunda regalis* using spores taken from *exsiccata*, providing new information on the viability of chlorophyllous spores, and useful data to further research on the best conditions and procedures for long-term conservation in germplasm banks. The germination of 17 and five year old chlorophyllous spores provides valuable information, especially when compared with previous studies

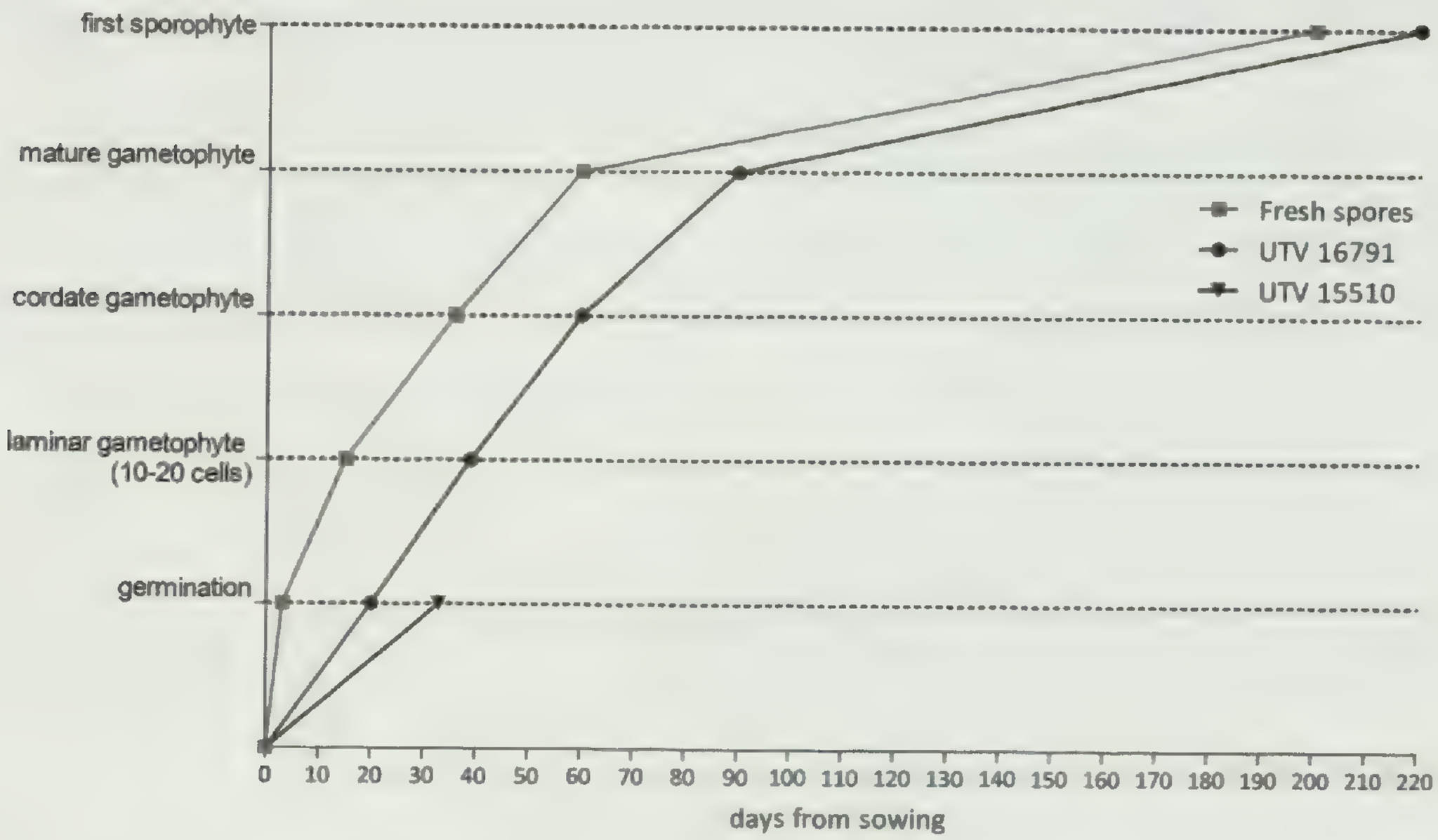

FIG. 1. Trend analysis of the *in vitro* reproduction of *Osmunda regalis*: the time of germination and subsequent development phases of the reproductive cycle.

(Lagerberg, 1908; Gerhardt, 1927; Okada, 1929; Lloyd and Klekowski, 1970) that provide germination data after a maximum of 150 days of storage at room temperature. Spore germination and subsequent sporophyte development from the five year old *exsiccata* could not be the product of cross contamination from newer *exsiccata* placed in the herbarium, because the specimen UTV 16791 of 2001 was the most recent specimen that had fertile fronds.

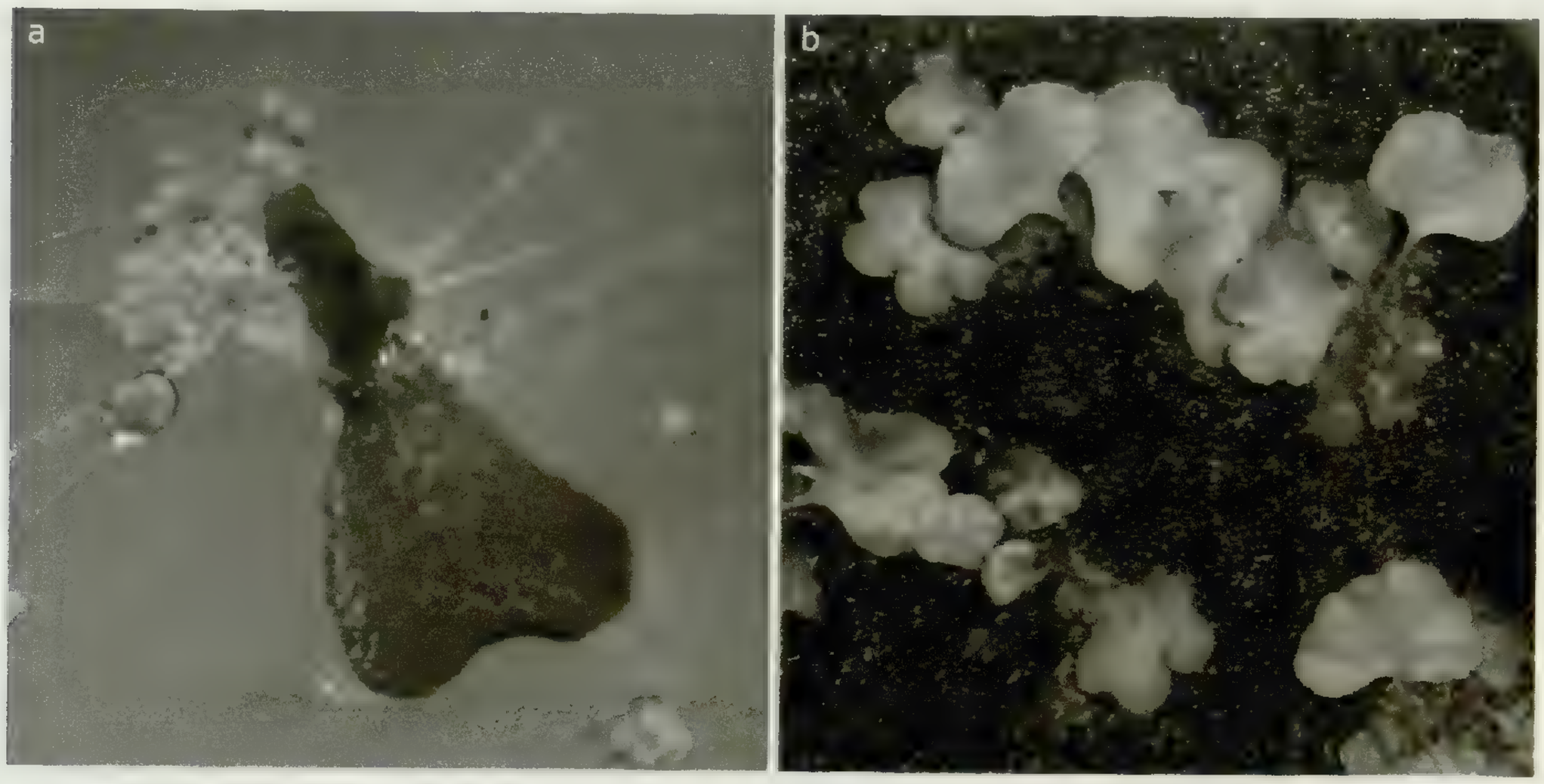

FIG. 2. **(a)** Young gametophyte with apical notch and **(b)** first leaves of the sporophytes obtained by spores collected from the *exsiccatum* UTV 16791 of 05/17/2001 (5 years old).

The viability of the spores of the *Osmundaceae* is considered to be of short duration. Lagerberg (1908) showed that the spores of *Osmunda regalis*, stored at room temperature, may be viable for two months, and Okada (1929) reported that the spores of certain *Osmunda* species are viable for a period of days (*Osmunda cinnamomea* L. for 43–54 days and *Osmunda japonica* Thunb. for 23–43 days). Furthermore, Gerhardt (1927) found that germination time increased with the age of the spores: spores three days old have germinated in one day; 34 days old in four days; 130 days old in seven days; 150 days old in 20 days; and spores 225 days old did not germinate. Our data confirm that the time necessary for germination is directly correlated with the age of spores (Lloyd and Klekowski, 1970), but that the development times of gametophytes and sporophytes do not show any correlation with spore age.

These results provide additional value to the herbarium *exsiccata* for conservation purposes. Herbaria are repositories not only of dried specimens for morphological or taxonomical studies, but are also a source of DNA for phylogenetic research (Rogers and Bendich, 1985; Cano and Poinar, 1993; Savolainen *et al.*, 1995; Cozzolino *et al.*, 2007), and even of living germplasm which have significant potential in the recovery of rare or endangered plant species, including lost genotypes, self-incompatible species, or those subject to inbreeding depression. Use of herbarium spores may be critical for restoring reproduction and/or genetic diversity in small or restored populations (Bowles *et al.*, 1993). This can be a useful method to reproduce species that are threatened or extinct in the wild, at least at a local level, so as to plan the re-introductions using the native germplasm (Lledó *et al.*, 1996; RENPA, 2007).

Herbaria are nonrenewable resources of germplasm of extinct or endangered species that could be appointed to special storage programs which would help to maximize longevity of spores using freezing treatments, but avoiding curatorial techniques like microwaving, excessive heat treatment or pesticides that cause abrupt loss of germplasm viability along with potential changes in plant morphology (Hill, 1983; Bacci *et al.*, 1985; Windham *et al.*, 1986). The spores taken from *exsiccata* and used for our tests were dehydrated and treated with four cycles of freezing and thawing, that are the conventional disinfestation treatments in UTV, so exposure to low temperatures seems to have little effect on spore viability.

We are continuing research on the development of tests with spores from various herbaria (CAG, FI, SIENA), selected from *exsiccata* with different age, chosen at regular intervals, to confirm the correlation between age and longevity of chlorophyllous spores under the same disinfestation treatments.

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American Fern Journal 100(3):167–171 (2010)

Growth and Care Instructions of a New Model Species—the Lycophyte *Selaginella apoda*

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ABSTRACT.—*Selaginella apoda* has many advantages as a model species, including a short life cycle. In order to work effectively with a lycophyte model species, it is important to be able to grow the gametophytes and sporophytes quickly. In the case of *S. apoda*, the gametophytes grow quickly and have high fertilization success in warm nutrient enriched water. Sporophytes at the three root stage can be easily transplanted to soil. They grow well in moist containers at a wide range of temperatures. Treatment with 0.15% Previcur®N prevents most fungal contaminations.

KEY WORDS.—model organism, lycophytes, lycopods, ferns, cultivation

Selaginella apoda (L.) Spring has many advantages as a model species, including an 85 day life cycle from spore to spore (Schulz *et al.*, 2010). The length of the life cycle is similar to other model species, such as *Ceratopteris thalictroides* (L.) Brongn., with a 3.5 month life cycle (Klekowski, 1970). In order to work effectively with a model species, it is important to be able to grow it quickly. In addition, a high frequency of fertilization is desirable.

Many different methods of growing ferns have been published (e.g., Lovis, 1968; Hickok and Warne, 2004), but only a small amount is known about growing *Selaginella*. Methods for growing gametophytes and sporophytes of *Selaginella* have been described, but the conditions and treatments vary among species. Bierhorst (1964) collected megaspores from the soil around *Selaginella* sporophytes and placed them on wet filter paper, where the spore wall opened. For the microgametophytes, he sowed whole microsporangia on filter paper. Bold (1967) sowed spores on plaster of Paris blocks half immersed in distilled water or an inorganic medium. If the plaster blocks remained sufficiently moist, fertilization would occur. Overall, little is known about growing *S. apoda*, therefore, we have undertaken this investigation to present an instruction on how to grow gametophytes and sporophytes of *S. apoda*.

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Materials and Methods

The specimens investigated here were collected from a naturally occurring population on the grounds of the New York Botanical Garden (population voucher: *Little & Moran 935*, NY). We grew specimens in the Nolen Greenhouse (New York, USA), in the greenhouse at the Botanical Garden of the Ruhr-University Bochum (Germany), and in laboratory conditions both in New York (USA) and Bochum (Germany). We grew *Selaginella apoda* six times through its entire life cycle. Spores were soaked in autoclaved water for 4 minutes, surface sterilized for 4 minutes, and then rinsed with autoclaved water for 4 minutes. For surface sterilization, the fungicides Benomyl (1–20 mg/L), sodium hypochlorite (0.1–0.875%), Previcur®N (propamocarb–hydrochlorite) (0.15%), and ethanol (70%) were tested. In addition, the antibiotic streptomycin (5–60 mg/L) was tested. Soil, sandy soil, peat soil, autoclaved water, autoclaved water with nutrients (C-fern nutrients) and C-fern agar (Carolina) were each used as media. The composition of these nutrient mixtures was the same as used by Klekowski (1969).

Results and Discussion

There are, theoretically of course, many possible protocols to grow *S. apoda* under different conditions. The method presented here is just one possibility, but it is very fast and successful. Masses of megaspores and microspores can be collected from pots of *S. apoda* (sporophytes with cones) by placing the pots on their sides on top of a smooth collecting surface (we used a sheet protector; Avery Denniso, Brea, CA; Fig. 1A). The greatest number and largest cones appeared in late summer and autumn (in New York). The cones produced in spring and early summer are fewer in number and much shorter. Presumably, cone and, therefore, spore production are influenced by day length and light intensity, but we have not investigated these parameters independently. In order to harvest a greater volume of spores, let the plants dry for one day during the collection, being careful not to dry the plants so much that they die. The collected spores can be stored in dry vials at room temperature; viability is maintained for at least two years. Growth can be accelerated by soaking the spores in water (or nutrient water) for 15 minutes before sowing.

Spore surface sterilization was necessary to grow sterile gametophyte cultures on used agar (Fig. 1 B, C). Biological contamination rarely occurred when soil or autoclaved water with or without nutrients was used as a medium; however, surface sterilization was necessary when using C-fern agar as a substrate. It is important to soak the spores with autoclaved water and to rinse the spores with autoclaved water after the treatment. Even a low concentration of 0.1% sodium hypochlorite, as routinely used for *Ceratopteris* spores (Hickok and Warne, 2004), killed the *S. apoda* megaspores. Sodium hypochlorite prevented only a small amount of fungal contamination. Both low concentrations of Benomyl and 70% ethanol also killed megaspores and prevented only some of the fungal contamination. In contrast, 0.15%

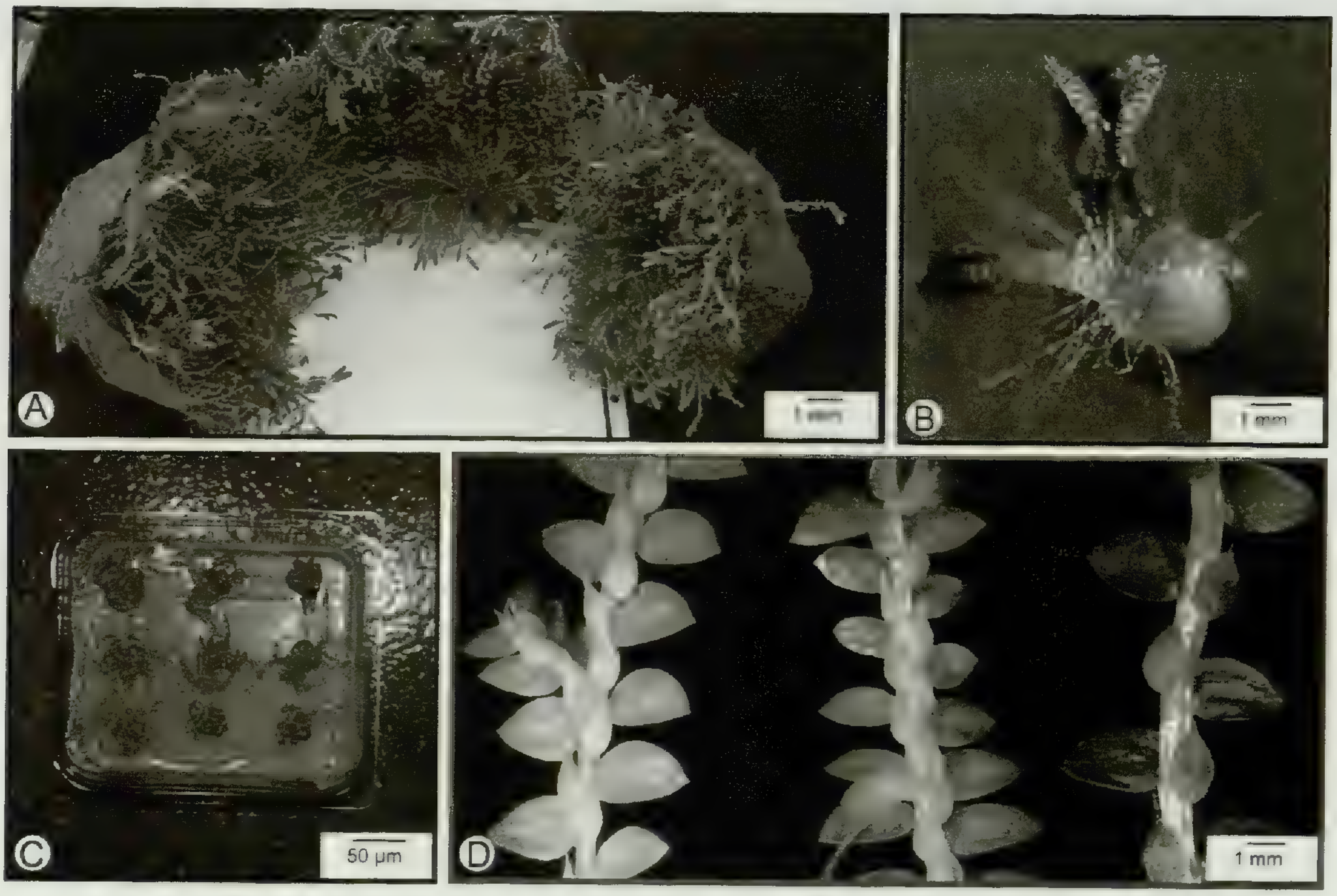

FIG. 1. *Selaginella apoda*. A: the pots with sporophytes arranged laterally on a smooth surface to collect mega- and microspores. B: young sporophyte growing on agar with Previcur®N. C: growth of sporophytes in a culture box. D: left: sporophytes growing in a bright place; the leaves are almost white; middle: sporophytes growing in a darker place than the sporophyte left but in a lighter place than the sporophyte right, the leaves are light green; right: sporophytes growing in a half shady place, the leaves are green.

Previcur®N prevented most fungal contaminations without killing the megaspores. Rarely, bacterial contamination occurred on C-fern agar, but 15 mg/L streptomycin (4 min) prevented such contamination. In order to prevent contamination with algae, it was sufficient to remove megaspores with algae (easily observed as green dots) from the culture.

The fertilization success varied with substrate. We observed the highest fertilization success (about 95%) in autoclaved water with nutrients. In autoclaved water and on C-fern agar (60–80%) fertilization was less frequent. On soil, the fertilization success was moderate (about 60%: Table 1).

An important factor for gametophyte development is temperature (Webster, 1967). The optimum temperature for gametophytic development is about 30°C. Similar growth and development of gametophytes can be obtained at 22–30°C. The higher the temperature within this range, the faster the gametophytes develop. Temperatures lower than 22°C will alter or inhibit the developmental timing. Light is not necessary for the development of the gametophytes. Gametophytes develop successfully under a 14h light/10h dark cycle as well as without any light (24h dark cycle) and in/on different kinds of media/ substrates.

TABLE 1. Number of developing sporophytes on different substrate/medium after different surface sterilization.

Substrate/ medium	Surface sterilization	Development	Fungal contamination	Bacterial contamination	Fertilization success
soil	none	++	−	−	+
water	none	++	−	−	++
water with nutrients	none	+++	+	+	+++
water with nutrients	0.15% Previcur®N	+++	−	−	+++
C–fern agar	none	++	+++	+++	+
C–fern agar	0.1% sodium hypochlorite	−	++	++	−
C–fern agar	70% ethanol	−	+	+	−
C–fern agar	5 mg/L Benomyl	−	+	+	−
C–fern agar	0.15% Previcur®N	++	−	(+)	+(+)
C–fern agar	0.15% Previcur®N, 15 mg/L streptomycin	++	−	−	+(+)

+ = 1–20%, ++ = 21–60%, +++ = 60–100%, (+) = 1–5%, +(+) = 10–40%

Webster (1979) described *S. apoda* growing in finely sifted sandy loam. Bruchmann (1912) indicated that peat is not suitable for the growth of *Selaginella* gametophytes as it has an inhibiting effect on *S. denticulata* (L.) Spring. However, we were able to grow *S. apoda* on peat soil (e.g., on peat pellets), on mineral agar (e.g., C-fern agar), in mineral medium (e.g., C-fern medium) or in tap water. For experimental purposes, a single megaspore and a single microspore develop very well in a 200 µL tube with 20 µL of water. It is important that the gametophytes are always covered with a film of warm water in order to prevent retardation of development and for fertilization success (the spermatozoids must be able to swim to the egg cells). If young sporophytes are grown in deep water, they usually produce elongated hypocotyls.

The optimal stage to transplant young sporophytes grown on agar (Fig. 1C) or in medium to soil is when the first three roots and several leaves are evident. The mineral nutrient medium used for gametophytes is not optimum for sporophytic growth, so transfer to a soil medium is preferable. Soil should be kept moist to wet at all times. Peat soil or sandy peat works well for *S. apoda*. The plants require warm temperatures (15–28°C) and high humidity (70–100%) to grow quickly.

The sporophytes grew very well at temperatures between 15°C and 28°C. The sporophytes grew faster at higher temperatures and produced more roots and rhizophores. Room temperature works fine for sporophytes. *Selaginella apoda* naturally lives in partially shaded habitats without direct sun. The more light the plants receive, the more the leaves turn yellow/white (i.e., are photobleached: Fig. 1D), and the faster the cones develop. With too much light, the plants will die. The less light the plants receive, the greener and smaller the leaves become. The optimal habitat is shady. Sporophytes grow fast in peat soil, and a bit slower on agar or in (nutrient) water ($n > 100$). If the

plants grow in soil, it is useful to use clay pots and keep the soil wet. The sporophytes need high humidity, otherwise the leaves dry out and the plant will die. The plants can be grown easily in a covered terrarium. The fungicide 0.15% Previcur®N (propamocarb–hydrochlorite) worked well against fungal contamination; it also works well with other *Selaginella* species. High concentrations of other fungicides can damage the plants.

In summer or under artificial light, when plants are growing very fast, fertilizer may be used. A low concentration of fertilizer is required for some *Selaginella* species. We achieved good growth and cone production with Compo® Orchid fertilizer (3–4–5–0.9 N–P–K–micronutrience) [Comp GmbH & Co. KG, Münster]. The recommended concentration should be applied once a month.

Acknowledgments

We thank Marc Hachadourian and the team of the Nolen Greenhouse for taking good care of *Selaginella apoda*. We also thank Thomas Stützel, Nina Minkley, Sabine Adler, Patrick Knopf, and Eric Brenner for their well-informed advice and for linguistic support. This research was supported by the National Science Foundation grant DBI-0421604. Additionally, we thank the reviewers for many helpful comments and improvements on the manuscript.

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American Fern Journal 100(3):172–179 (2010)

New Species and New Records in *Elaphoglossum* sect. *Polytrichia* subsect. *Hybrida* (Dryopteridaceae) from the Neotropics

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ABSTRACT.—Three new species of *Elaphoglossum* sect. *Polytrichia* subsect. *Hybrida* from the Neotropics are described here: *E. lucens*, *E. martinezianum*, and *E. reductum*. The first species is characterized by blackish, lustrous and rigid rhizome scales and short fertile fronds. The second species is characterized by stipe and blade margins with long scales and blades elliptic and abaxially scaly. The third species is easy to recognize by small and curved scales and obovate blades. New geographic records are reported for three species, thus expanding their ranges.

KEY WORDS.—Ferns, Neotropics, taxonomy, systematics

Mickel and Atehortúa (1980) characterized *Elaphoglossum* sect. *Polytrichia* subsect. *Hybrida* as having a short-creeping or ascending rhizome, long stipes, usually papyraceous blades, blade scales black or dark brown and distributed especially along the margin and costa, and spores with low ridges. The *Elaphoglossum erinaceum* (Fée) T. Moore complex has been considered difficult taxonomically because of its variation in blade shape and size, and size, color and habit of the rhizome scales (Mickel, 1991, 1992, 1995; Mickel and Beitel, 1988). Relatively little work has been done on the taxonomy of the section except for that of Rojas (2002, 2003) from the Neotropics.

As a result of my research on *Elaphoglossum*, I herein describe three new species belonging to the group of *E. erinaceum* and provide new distributional records for three species.

Elaphoglossum lucens A. Rojas, ***sp. nov.*** TYPE.—PERU. **Cajamarca**: San Ignacio Province, Distrito Huarango, El Convento, 5°13′S, 78°40′W, 1100–1400 m, 2 July 1996, *J. Campos & E. Rodríguez 2866* (holotype: MO). **Figs. 1A, 2.**

A Elaphoglosso kessleri A. *Rojas rhizomatis squamis brevioribus nigris lucens, frondibus fertilibus brevioribus differt.*

Epiphytic; rhizome 7–10 mm in diameter, compact, ascending; rhizome scales 2–5 × 0.3–0.6 mm, linear-lanceolate, dark brown to blackish, lustrous, rigid, marginally entire; fronds 20–34 cm long; stipe 4–9 cm long, 1/6–2/7 of the frond length, yellowish to stramineous, scaly, the scales 2.0–3.5 × 0.1–0.4 mm, subulate, dark brown to blackish, bulliform basally, sparse to medium-dense, marginally entire; phyllopodia 2–7 mm long; blade 15–29 × 1.1–2.7 cm, linear-elliptic, basally cuneate, apically acuminate; costa strami-

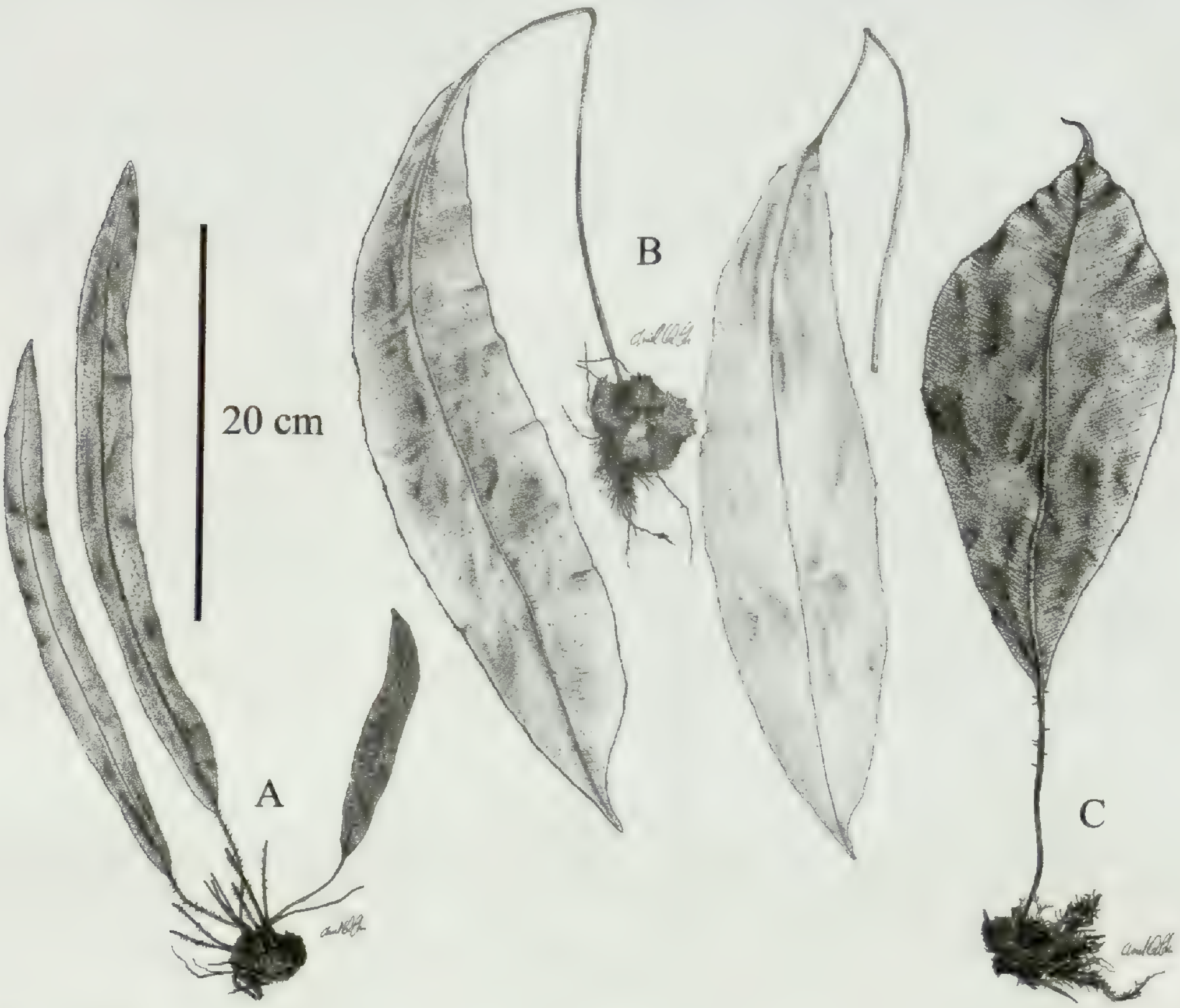

FIG. 1. Type specimen of: A. *Elaphoglossum lucens* (*J. Campos & E. Rodríguez 2866*, MO). B. *E. martinezianum* (*D. Breedlove & A. Smith 31630*, MEXU). C. *E. reductum* (*A. Rojas et al. 5043*, CR).

neous, scaly on both sides, the scales 1–2 × 0.2–0.5 mm, linear-lanceolate to linear, blackish, marginally entire to denticulate; margin scales 0.5–1.5 × 0.1–0.3 mm, linear-lanceolate, blackish; adaxial surface scaly, the scales similar to the margin ones; abaxial surface with two types of hairs, some ca. 0.1 mm, single to stellate, dark brown to blackish, appressed, the others less than 0.1 mm long, single, patent, hyaline, glandular, present on and near the costa; veins single to bifurcate near the base, 1–1.5 mm distant, at angles of 60–70° with respect to the costa; fertile fronds 10–19 cm long, shorter than the sterile; stipe 1/3–2/5 of the frond length; blade 6.0–9.5 × 1.3–1.5 cm; interesporangial scales absent.

DISTRIBUTION.—Known only from the type collection.

ETYMOLOGY.—The specific epithet of the new species refers to its lustrous rhizome scales.

Elaphoglossum lucens differs from *E. kessleri* A. Rojas by shorter (2–5 mm vs. 6–10 mm), dark brown to blackish (vs. yellowish to brown), lustrous (vs.

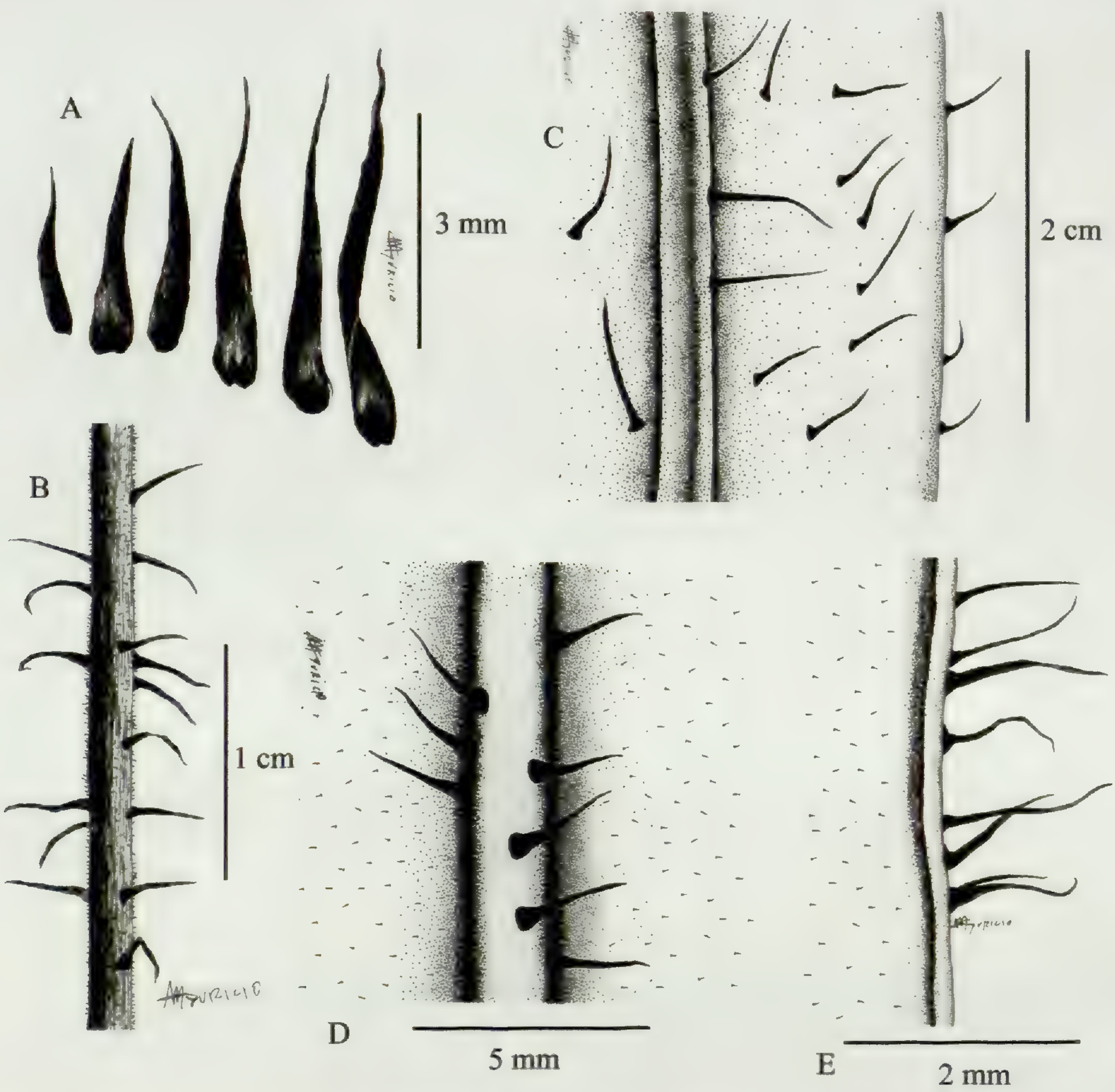

FIG. 2. *Elaphoglossum lucens* (*J. Campos & E. Rodríguez 2866*, MO): A. Rhizome scales. B. Stipe detail. C. Adaxial blade detail. D. Abaxial costa detail. E. Abaxial blade margin detail.

opaque) and rigid (vs. flaccid) rhizome scales, shorter (1/6–2/7 of the frond length vs. ca. 1/3) stipes and shorter (10–19 cm long vs. 25–37 cm) fertile fronds with relatively shorter (1/3–2/5 of the frond length vs. 2/5–2/3) stipes. It resembles *E. crispipalea* M. Kessler & Mickel but differs by shorter (2–5 mm vs. 5–9 mm), dark brown to blackish (vs. reddish-brown to orange), entire (vs. denticulate), flat (vs. with crispate margin) rhizome scales, and scaly (vs. glabrous) blades (Figs. 1A, 2).

Elaphoglossum martinezianum A. Rojas, ***sp. nov.*** TYPE.— MEXICO. **Chiapas**: SE side of Volcán Tacaná, above Talquian, 2200 m, 16 Jan 1973, *D. Breedlove & A. R. Smith 31630* (holotype: MEXU). **Figs. 1B, 3.**

A Elaphoglosso barbato (H. Karst.) Hieron. *lamina elliptica cum basi cuneata differt.*

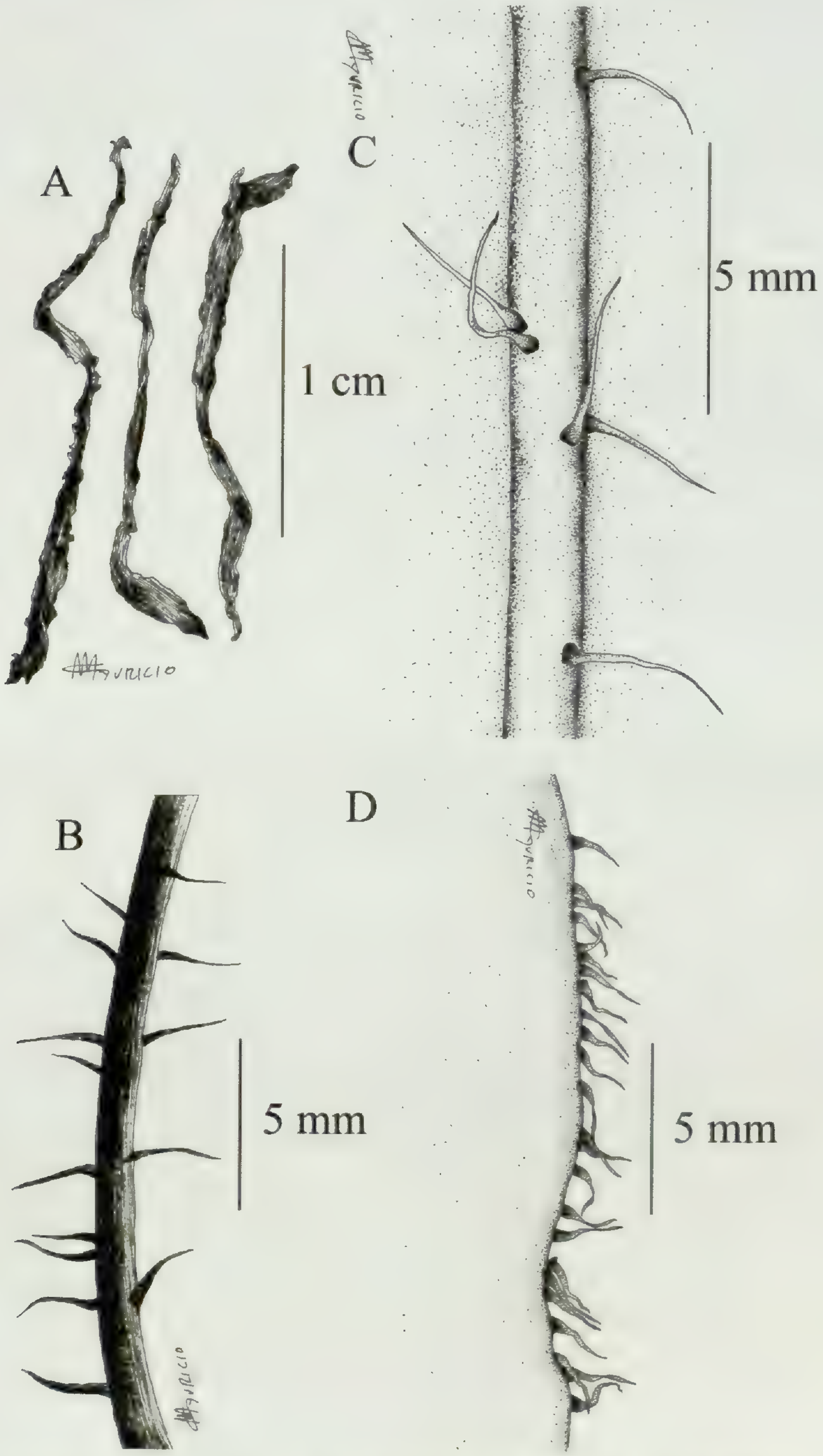

FIG. 3. *Elaphoglossum martinezianum* (*D. Breedlove & A. Smith 31630*, MEXU): A. Rhizome scales. B. Stipe detail. C. Abaxial costa detail. D. Adaxial blade margin detail.

Epiphytic; rhizome 6–8 mm in diameter, compact, ascending; rhizome scales 10–18 × ca. 1 mm, linear, orange-brown, opaque, flaccid, marginally entire; fronds 50–65 cm long; stipe 20,5.22.5 cm long, ca. 2/5 of the frond length, yellowish to stramoneous, scaly, the scales 1–3 × 0.2–0.5 mm, subulate, dark brown to blackish, buliform basally, sparse to dense, marginally entire; phyllopodia 4–7 mm long; blade 29.5–32 × 7.0–7.2 cm, elliptic, basally cuneate, apically acuminate; costa stramineous, scaly abaxially, the scales 2–4 × 0.2–0.5 mm, linear-lanceolate to linear, reddish-brown to blackish, marginally entire to denticulate; margin scales 1.5–2.0 × 0.1–0.3 mm, linear-lanceolate, reddish-brown to blackish; adaxial surface glabrous; abaxial surface scaly, the scales 1.5–3 × 0.1–0.3 mm, linear-lanceolate, blackish, appressed; veins single to bifurcate near the base, 1–1.5 mm distant, in an angle of 60–70° with respect to the costa; fertile fronds not seen.

DISTRIBUTION.—Known only from Chiapas, Mexico; 1550–2200 m.

ETYMOLOGY.—The specific epithet is dedicated to Marta Martínez Gordillo, Mexican botanist who helped me with my doctoral thesis.

PARATYPES.—MEXICO. **Chiapas**: Municipio Unión de Juárez, en el Volcán Tacaná, a 5 km al S de Talquián, 1550–1700 m, 8 Feb 1987, *E. Martínez et al. 19796* (MEXU).

Elaphoglossum martinezianum is similar to *E. barbatum* (H. Karst.) Hieron. by blades abaxially scaly, but it differs by longer (10–18 mm vs. 4–8 mm) rhizome scales and elliptic (vs. lanceolate to oblong) blades with cuneate (vs. obtuse to truncate) bases. Also similar is *E. mexicanum* (E. Fourn.) A. Rojas; however, *E. martinezianum* differs by stipe and blade margins less densely scaly, proportionaly longer (ca. 2/5 of the frond length vs. 1/5–1/3) stipe, broader (7.0–7.2 cm vs. 2.5–4.5 cm) blade and abaxialy scaly (vs. not scaly) (Figs. 1B, 3).

Elaphoglossum reductum A. Rojas, ***sp. nov.*** TYPE.—COSTA RICA. **Cartago**: Cantón de Turrialba, Reserva Indígena Chirripó, camino a Valle Escondido, entrando por Las Brisas de Pacuarito, entre casa de Don Felipe y Quebrada Olomina (límite del parque), 9°56′40″N, 83°28′40″W, 750–850 m, 13 Apr 1999, *A. Rojas et al. 5043* (holotype: CR; isotypes: INB, MO). **Figs. 1C, 4.**

A Elaphoglosso erinaceo (Fée) T. Moore *similis, rhizomatis squamis brevioribus, lamina ovobata differt.*

Epiphytic; rhizome 7–10 mm in diameter, compact, ascending; rhizome scales 0.5–2.0 × 0.3–0.5 mm, ovate to lanceolate, grayish-brown to brown, buliform to irregular, marginally entire; fronds 33–43 cm long; stipe 9–22 cm long, 1/4–1/2 of the frond length, yellowish to stramineous, scaly, the basal scales 1–3 × 0.5–1.0 mm, ovate to lanceolate, yellowish-brown, flat or curved (but no bulliform), marginally entire, the apical scales 3–6 × 0.2–0.5 mm, subulate, dark brown to blackish, sparse, marginally entire; phyllopodia 3–7 mm long; blade 24–26 × 10–11 cm, obovate, basally cuneate, apically

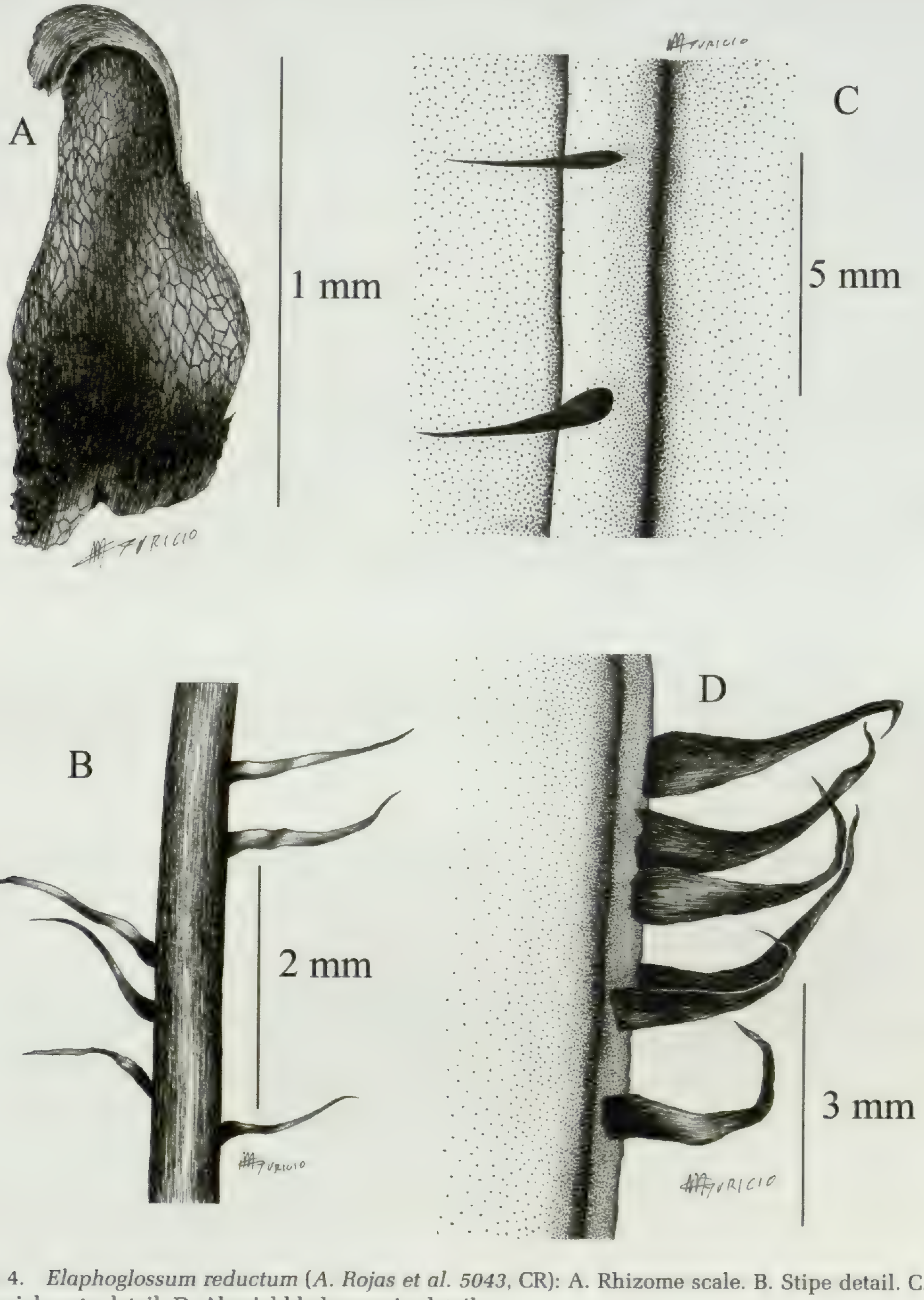

FIG. 4. *Elaphoglossum reductum* (*A. Rojas et al. 5043*, CR): A. Rhizome scale. B. Stipe detail. C. Abaxial costa detail. D. Abaxial blade margin detail.

acuminate; costa yellowish to stramineous, scaly, the scales similar to those of the stipe; marginal scales 1.5–3.0 × 0.2–0.4 mm, subulate, blackish; costa and abaxial surface with two types of hairs, some less than 0.1 mm in diameter, stellate, black, denser near the costa, the others less than 0.1 mm long, single,

patent, hyaline, glandular; veins single to bifurcate near the base, 1–1.5 mm distant, at angles of 65–75° with respect to the costa; fertile fronds not seen.

DISTRIBUTION.—Known only from the Fila Matama in the Caribbean side of Cordillera de Talamanca in Costa Rica; 720 m.

ETYMOLOGY.—The specific epithet of the new species refers to its smaller rhizome scales compared with those of *E. erinaceum*.

Elaphoglossum reductum differs from *E. erinaceum* (Fée) T. Moore in having smaller [0.5–2.0 × 0.3–0.5 mm vs. 8–17 × (0.8–) 1.5–3.5 mm], ovate (vs. linear) and grayish-brown to brown (vs. yellowish to orange) rhizome scales and broader (10–11 cm vs. 4–9 cm), and obovate (vs. elliptic) blades with to base cuneate (vs. long-decurrent). No species in subsect. *Hybrida* has smaller rhizome scales and obovate blades (Figs. 1C, 4).

NEW DISTRIBUTION:

Elaphoglossum angustioblongum A. Rojas, Revista Biol. Trop. 51: 34. 2003. TYPE.—PANAMÁ. **Chiriquí**: Las Cumbres, *T. Croat & D. Porter 16078* (holotype: MO!).

PREVIOUSLY KNOWN DISTRIBUTION.—Costa Rica and Panama.

MATERIAL OF NEW DISTRIBUTION.—MEXICO. **Chiapas**: Sierra Madre de Chiapas, ridge top along trail to E from high point on road between Finca Liquidambar and Nueva Colombia, ca. 15°40′N, 92°44′W, ca. 2500 m, 18 June 1985, *J. Luteyn & M. Lebrón 11601* (MEXU).

Elaphoglossum baquianorum A. Rojas, Revista Biol. Trop. 51: 33–48. 2003. Type: Costa Rica, Limón/Puntarenas, entre Cerro Kasir y Cerro Nai, 2940–3100 m, *A. Rojas et al. 3208* (holotype: INB; isotypes: CR, MO, NY, UC, US).

PREVIOUSLY KNOWN DISTRIBUTION.—Mexico, Guatemala, Honduras, El Salvador, Honduras, Nicaragua, Costa Rica, and Panama.

MATERIAL OF NEW DISTRIBUTION.—BOLIVIA. **La Paz**: Franz Tamayo, along trail between Pelechuco and Pata, along the Río Pelechuco down stream from Pelechuco, 14°46′S, 69°01′W, 3200 m, 16 Nov 1988, *M. Lewis 881732* (MO).

Elaphoglossum scolopendrifolium (Raddi) J. Sm., Bot. Mag. 72: 17. 1846. *Acrostichum scolopendrifolium* Raddi, Pl. Bras. 1: 4, t. 16. 1825. Type: Brazil. Rio de Janeiro: *Raddi s.n.* (holotype: PI, not seen).

PREVIOUSLY KNOWN DISTRIBUTION.—Mexico and Brazil.

MATERIAL OF NEW DISTRIBUTION.—PERU. **Amazonas**: Provincia of Bagua, ca. 12–17 km (by trail) E of La Peca, ca. 1700–2100 m, 28 June 1978, *P. Barbour 2528* (MO).

ACKNOWLEDGEMENTS

I thank the curators of the herbaria at the Museo Nacional de Costa Rica (CR), Instituto Nacional de Biodiversidad (INB), Instituto de Biología (MEXU), and Missouri Botanical Garden (MO) for loans of specimens and the anonymous reviewers for their helpful suggestions.

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ROJAS, A. F. 2003. Notes on *Elaphoglossum* (Lomariopsidaceae) section *Polytrichia* subsection *Hybrida* in Mexico and Central America. Rev. Biol. Trop. 51:33–48.

American Fern Journal 100(3):180–187 (2010)

Shorter Notes

***Hymenophyllum pilosissimum* C. Chr. (Hymenophyllaceae), a New Recorded Fern from Taiwan.**—Taiwan is one of the most floristically rich areas in the world in its fern composition, which comprise at least 633 species and infraspecific taxa. This kind of high diversity could be attributed to several different reasons including warm and wet weather, altitudinal variation, diverse habitats, and geographical and historical factors (Moore, Diversity and conservation of Taiwan pteridophytes. In: Chow *et al.*, eds., Proceedings of the 2000 cross-strait symposium on biodiversity and conservation pp.331–359. National Museum of Natural Science, Taichung. 2000). Due to the complex topography of Taiwan, many places have never or seldom been visited by fern collectors. This situation has improved because of increased interest in biological diversity by the government and academic sectors since the issue of the Flora of Taiwan (Huang, ed.-in-chief, Flora of Taiwan. 2nd. ed. Vol. I. Editorial Committee of the Flora of Taiwan, Second Edition, Taipei. 1994), and some new recorded ferns and lycophytes were discovered in recent years (e.g., Moore *et al.*, Taiwan J. For. Sci. 17:113–118. 2002; Chang *et al.*, Taiwania 48:197–202. 2003; Moore *et al.*, Taiwania, 48:22–28. 2003; Ebihara *et al.*, Taxon 53:935–948. 2004; Lu and Yang, Taiwania 50:137–165. 2005; Liu and Fraser-Jenkins, Taiwania 51:293–297. 2006; Shinohara *et al.*, Amer. Fern J. 96:96–99. 2006; Chang *et al.*, Taiwania 51:287–292. 2006; Chang *et al.*, Taiwania 52:238–242. 2007; Liu *et al.*, Amer. Fern J. 97:166–173. 2007; Chang *et al.*, Taiwania 54:88–92. 2009; Liu *et al.*, Novon 19:59–61. 2009).

In this article we present another unreported filmy fern, *Hymenophyllum pilosissimum* C. Chr. (Hymenophyllaceae), for the flora of Taiwan. Its taxonomic treatment, line drawings, ecology, phytogeography, and conservation status are reviewed here.

Hymenophyllum pilosissimum C. Chr. in C. Chr. and R. E. Holttum, Gardens' Bull. Straits Settlem. 7: 213. 1934. (Type: Borneo, Kinabalu, *Burbidge s.n.* K!) (**Fig. 1**).

Sphaerocionium pilosissimum (C. Chr.) Copel. Philipp. J. Sci. 67: 33. 1938.

Plant delicate, epiphytic or lithophytic. Rhizomes long-creeping, wiry, widely branched, rootless, but with root-like shoots, terete, glabrous when old, sparsely to fairly hairy when young; rhizomal hairs light-brown, stalked, usually with 1–2 distal branches, hardly to 3 branches, stalks ca. 0.1–0.35 mm long, branches ca. 0.25–0.55 mm long. Fronds 2–14 cm long, 0.8–3.5 cm wide; stipes 0.5–3.5 cm long, wingless on most part, terete, hairy; stipe hairs similar to those on laminae, but only with 2–4 distal branches; laminae 2-pinnate to 3-pinnate-pinnatifid, variable in shape, elliptic, lanceolate, oblanceolate, narrowly lanceolate, narrowly oblanceolate to narrowly elliptic; ultimate

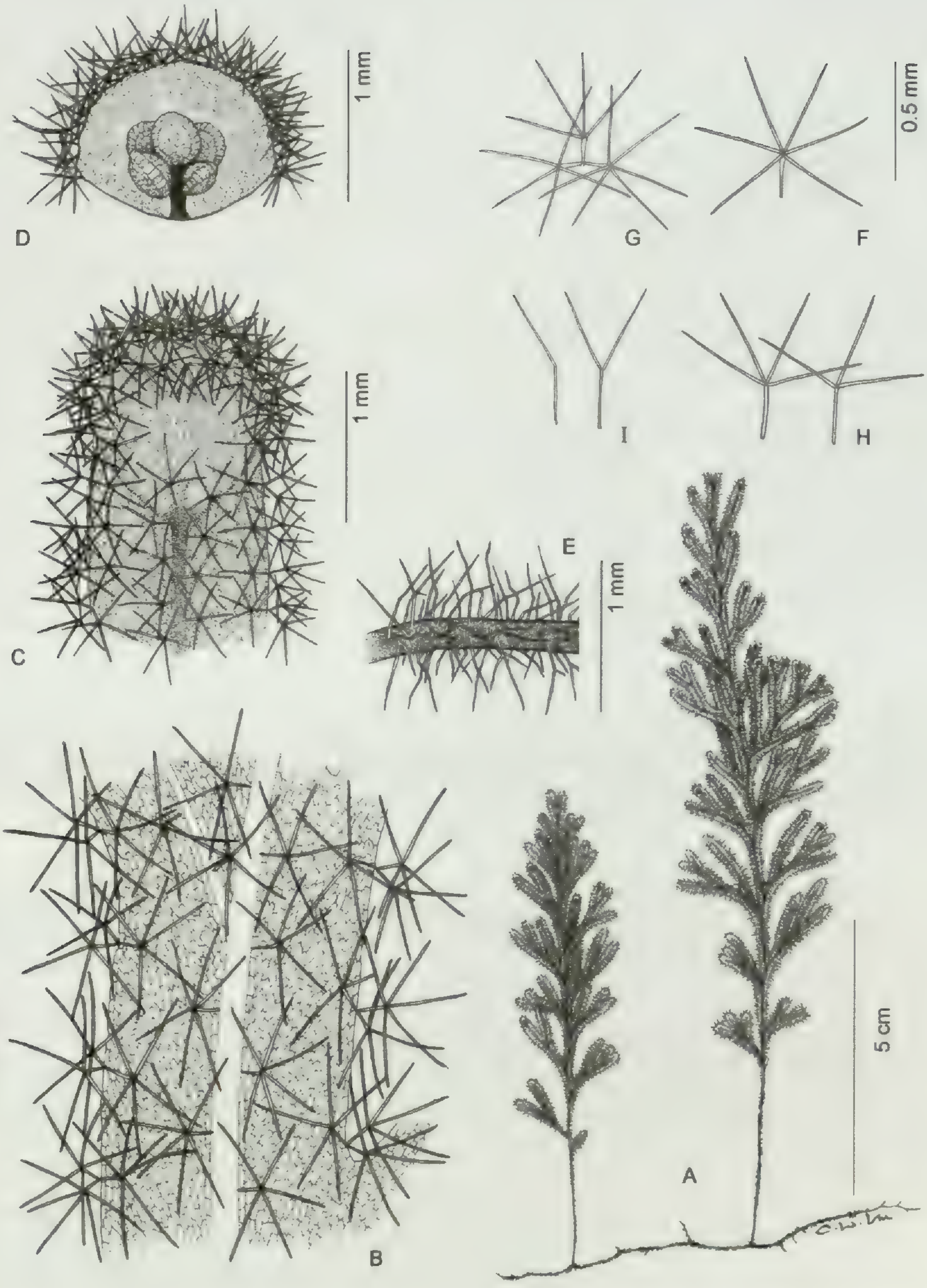

FIG. 1. *Hymenophyllum pilosissimum*: A. habit; B. adaxial surface of lamina; C. fertile segment; D. sorus; E. rhizome; F. simple stellate hair with a stalk on vein; G. 3-tufted stellate hair on laminar margin; H. stellate hairs with fewer branches from stipe; I. hairs from rhizome (all from *Chen s. n.*, TAIF).

pinnules 0.8–1.3 mm wide; laminar hairs light-brown, stellate, with a stalk and 4–6 distal branches, abundant on veins and margins, usually simple stellate hair (Fig. 1F) on veins, 3-tufted stellate hair on margins (Fig. 1G), absent from laminal surfaces, stalks ca. 0.12–0.4 mm long, branches ca. 0.2–0.8 mm long.

Sori on the tips of the upper ultimate segments; indusium bivalate on upper part, with simple, forked or 3-tufted, stalked, stellate hairs on lip margins; receptacle short, included, not protruding.

SPECIMENS EXAMINED.—TAIWAN. **Pingtung/Taitung Hsien:** Chinshuiying to Mt. Kutzulunshan, 22°25′N, 120°46′E, 1500 m, *S. J. Moore 19632,* 23 June, 1997 (TAIF, TNU). **Taipei Hsien**: Wantan, 24°56′N, 121°48′E, 550 m, *C. M. Chen, s. n.,* 25 Aug. 2003 (TAIF).

NOTES.—*Hymenophyllum pilosissimum* is very similar to *H. obtusum* (Hook. & Am.) Copel., a Hawaiian endemic species, but can be distinguished by their laminar hairs, usually with 4–6 distal spreading branches in the former and with 2–3 ascending branches in the latter. It is also similar to the New Caledonian endemic species, *H. subobtusum* Rosenst., but the latter has 3-tufted stellate hairs on veins and wider ultimate pinnules (up to 2 mm).

This species is often placed in genus *Sphaerocionium* (e.g., Copeland, Philipp. J. Sci. 67:1–100. 1938, Fern flora of the Philippines. Vol. I. Bureau of Printing, Manila. 1958; Iwatsuki, J. Fac. Sci. Univ. Tokyo, Sect. 3, Bot. 13:203–215. 1982, J. Fac. Sci. Univ. Tokyo, Sect. 3, Bot. 13:501–551. 1985; Parris *et al.*, The plants of Mount Kinabalu I. ferns and fern allies. Royal Botanic Gardens, Kew. 165pp. 1992). Here we follow Ebihara *et al.* (Blumea 51:221–280. 2006), adopt *Hymenophyllum* as the genus name for this species because of the good support from molecular evidence.

In the protologue, Christensen and Holttum (Gard. Bull. Straits Settlem. 7:191–324. 1934) stated: "The undersurface is throughout covered with stellate hairs which bear....". However, according to the type and our specimens, laminar hairs are confined to the margins and veins. The distribution of hairs on lamina was also confirmed by Iwatsuki (1982), who treated all species with the same characteristic under *Sphaerocionium* subsect. *Sphaerocionium*.

Only two populations were found in northern and southern Taiwan (Fig. 2). The southern population (Pingtung/Taitung) was found on mossy tree trunks of cloud forests at 1,400–1,800 m of elevation. Their fronds are relatively small, usually less than 3.5 cm long, possibly stunted because of the common occurrence of strong winds. The northern population (Taipei) was discovered on rocks on slopes of wet forests of 400–700 m elevation, usually with fronds >5 cm in length.

The species is mainly distributed in Malaysia, including the Philippines (Christensen and Holttum, 1934; Copeland, 1958), Borneo (Christensen and Holttum, 1934; Parris *et al.*, 1992; Ebihara, Mem. Natl. Mus. Nat. Sci. 45:105–110. 2008), and New Guinea (Christensen and Holttum, 1934; Iwatsuki, 1982). The distribution of this species in Taiwan represents its northernmost range.

Hymenophyllum pilosissimum is widely distributed from New Guinea to Taiwan, therefore it should not be regarded as a rare species worldwide. Nevertheless it is a locally rare species in Taiwan. Owing to this rarity, it should be considered a species of concern for conservation, and we suggest that a rank of vulnerable (VU D1 + 2) be assigned to it for Taiwan under the IUCN Red List criteria (IUCN, IUCN Red List Categories and Criteria, Version 3.1. IUCN Species Survival Commission, IUCN, Gland, Switzerland, and

FIG. 2. Distribution map.

Cambridge, UK. 2001) and the guidelines for application of IUCN Red List criteria at regional levels (IUCN, Guidelines for application of IUCN Red List criteria at regional levels: Version 3.0. Species Survival Commission, IUCN, Gland, Switzerland, and Cambridge, UK. 2003).

The authors thank Dr. Yea-Chen Liu and the workers of K, KYO, TAIF, and TNU for helping examination of specimens and Mr. Che-Wei Lin for the illustration of the voucher specimen.—SHANN-JYE MOORE, Department of Life Science, National Taiwan Normal University, 88, Ting-Chow Rd., Sec 4, Taipei 11677, Taiwan, CHUN-MING CHEN, Dr. Cecilia Koo Botanic Conservation Center, Ping-Tung, Taiwan, and JENN-CHE WANG, Department of Life Science, National Taiwan Normal University, 88, Ting-Chow Rd., Sec 4, Taipei 11677, Taiwan, E-mail: biofv017@ntnu.edu.tw.

New Records of Ferns from Northeastern Argentina and Uruguay.—Four records of ferns are reported here for the first time, two species for northeastern Argentina and two others for Uruguay. In addition, the occurrence of other species is documented with herbarium specimens for both countries. The new distribution records are supported by recent collections done by the authors

and by specimens studied at CTES Herbarium. The collections that document the distribution extensions for Argentina come from Misiones. This area belongs to the Amazonian domain (Cabrera and Willink, Biogeografía de América Latina, Serie Biología 13, Secretaría de la OEA, pp. 117. Washington D.C. 1973), and it represents the greatest center of pteridophyte diversity in Argentina (Ponce *et al.*, Revista Chilena Hist. Nat. 75: 703–717. 2002).

Uruguay is inside the "Chaqueño" phytogeographical domain, in the "Pampeana" province of Cabrera and Willink (1973), which is characterized principally by grass prairies. This country presents a high level of diversity of ligneous species, probably due to high variability of environments across short distances, and also because of sharing a connection with Brazil's southern flora. The floristic connection raises the possibility that many species should reach the limit of their natural distributions in Uruguay (Brussa and Grela, Flora arbórea del Uruguay. Con énfasis en las especies de Rivera y Tacuarembó, pp. 65, COFUSA, Montevideo. 2007).

Since the works published by Osten and Herter (Anales Mus. Hist. Nat. Montevideo ser. 2, 3:109–256. 1925) and Legrand and Lombardo (Flora del Uruguay I, Pteridophyta, Museo Nacional de Historia Natural, Montevideo, pp. 67. 1958), few contributions have been made to the pteridophyte flora of Uruguay. The Catalogue of Vascular Plants of the Southern Cone edited by Zuloaga, Morrone and Belgrano (Monogr. Syst. Bot. Missouri Bot. Gard., vol. 1. 2008) overlooks part of the diversity of ferns and lycophytes present in this country; with this note, a small step is taken to fill in this gap.

Deparia petersenii (Kunze) M. Kato, Bot. Mag. (Tokyo) 90(1017):37. 1977.

Asplenium petersenii Kunze, Analecta Pteridogr. 24. 1837. *Diplazium petersenii* (Kunze) H. Christ, Bull. Acad. Int. Géogr. Bot. 245. 1902.

DESCRIPTIONS AND ICONOGRAPHIES.—Sehnem, Aspidiáceas, 307–308, f. 75, (*sub nomen D. intercalatum* H. Christ) in Reitz (ed.), *Flora Ilustrada Catarinense* Parte I (ASPI) Itajaí, SC, Brasil. 1979; Cislinski, J. (Acta Bot. Bras. 10(1): 67, f. 2 A–C, 5 C, 6 C. 1996).

DISTRIBUTION AND COMMENTARIES.—This fern grows in Asia, Pacific Islands, and Australia, and it was introduced in southeastern United States (Kato, *Diplazium* Sw. and *Deparia* Hook. & Grev., pp. 254–255, in Flora of North America Editorial Committee, Flora of North America. vol. 2. Oxford University, N.Y. 1993). In South America, the species was cited by Cislinski (1996) for states of southeastern and southern Brazil. The species is reported here for the first time from Argentina, where it was collected on a rocky slope by the side of a road in Misiones.

This fern, with blade pinnate-pinnatifid, is characterized by possessing multicellular hairs on both sides of the leaves (Cislinski, 1996).

Kato's treatment of *Diplazium* and *Deparia* (1993) considered both genera as separate and distinct, justifying this separation based on the non-decurrent

costal grooves and the presence of multicellular hairs on blades in *Deparia*. This treatment is accepted here.

SPECIMEN STUDIED: Argentina, Misiones, Dpto. Montecarlo, Ruta Nacional 12, S26°40′36.9″, W54°47′07.8″, 1-IX-2008, *Keller 5943* (CTES).

Doryopteris collina (Raddi) J. Sm., J. Bot. (Hooker) 4: 163. 1841.

DESCRIPTIONS AND ICONOGRAPHIES: Sehnem, Pteridáceas, 161–162. f. 38–2, *in* Reitz (ed.), *Flora Ilustrada Catarinense* Parte I (PTER) Itajaí, S.C., Brasil. 1972.

DISTRIBUTION AND COMMENTARIES: This taxon is distributed in Trinidad, Guianas, Paraguay and different states from the northeastern, southeastern and southern regions of Brazil (Pichi Sermolli & Bizzarri, Webbia 60 (1): 226–228. 2005). With this new record, eight species of *Doryopteris* are represented in Argentina. The studied specimen represents the only known population for this country and was collected on rocky slopes inside forest.

As *Doryopteris concolor* (Langsd. & Fisch.) Kuhn, *D. collina* is separated from other species of *Doryopteris* J. Sm. present in Argentina by the wing-angled petiole, but with a less divided lamina.

SPECIMEN STUDIED: Argentina, Misiones, Dpto. San Ignacio, Club del Río, 8-II-2009, *Keller & Paredes 6530* (CTES).

Ophioglossum reticulatum L., Sp. Pl. 2: 1063. 1753.

DESCRIPTIONS AND ICONOGRAPHIES: Sehnem, Ophioglóssaceas, 4–6, f. 1–6, *in* Reitz (ed.), *Flora Ilustrada Catarinense* Parte I (OFIO) Itajaí, S.C., Brasil. 1979.

DISTRIBUTION AND COMMENTARIES: This species presents a tropical and subtropical distribution in both hemispheres. It is known in Africa, Asia, Australia, and in America it grows in the southwest of the United States (*sub nomen Ophioglossum petiolatum* Hook., Wagner & Wagner, Ophioglossaceae, pp. 85–106, *in* Flora of North America Editorial Committee.1993), Mexico, Central and South America to Paraguay, Brazil, and Argentina (Mickel and Smith, Memoirs of The New York Botanical Garden 88:432–433. 2004). The most southern population of the Southern Cone was registered in the delta of the Paraná River, in Buenos Aires, near the latitude of 34°22′ S. This taxon grows in disturbed or moderately disturbed places, generally in the herbaceous layer in forests degraded by pasturing or in the woody edges, but always in the protection of a shrubby or arboreal layer. However, it can also inhabit pastures, commonly dominated by *Andropogon*, since these grasses afford the fern some protection.

The population in Uruguay mentioned in this note was in the border of a plantation of *Pinus elliottii* Engelm., growing among a layer of pine needles,

next to inundated soil. *Ophioglossum reticulatum* is distinguished from the other two present species in Uruguay, *O. crotalophoroides* Walter and *O. nudicaule* L *f.*, for its epigeous petioles up to 10 cm in length; its cordate blade, and cylindrical rhizome. The other two species possess underground petioles of less than 6 cm in length, blades subcordate and ovate, and rhizomes globose and subcylindrical, respectively.

SPECIMEN STUDIED: Uruguay, Dpto. Rivera, Cofusa, Estableciemento Batoví, Arroyo Batoví, S 31°02′08″, W 55°24′46.20″, *Brussa & Grela s.n.* (MNJB 23211).

Serpocaulon catharinae (Langsd. & Fisch.) A. R. Sm., Taxon 55(4):928. 2006.

Polypodium catharinae Langsd. & Fisch., Pl. Voy. Russes Monde 1, t. 9. 1810.

Goniophlebium catharinae (Langsd. & Fisch.) J. Sm. in Hook., Gen. Fil. [Hooker], t. 51. 1840.

DESCRIPTIONS AND ICONOGRAPHIES: Hensen, Nova Hedwigia 50 (3–4):292–293, fig. 9, 38–39, 64. 1990; Labiak & Prado, Bol. Inst. Bot. (Sao Paulo) 11:51, fig. 7 P. 1998; Sehnem, Polipodiáceae, 105–109, f. 33, in Reitz (ed.) *Flora Ilustrada Catarinense* Parte I (POLI) Itajaí, SC, Brasil, 1970 (all *sub nomen P. catharinae*).

DISTRIBUTION AND COMMENTARIES: This species was mentioned from Bahia, and seven states of southern and south of Brazil, Uruguay, Argentina, and Paraguay (Labiak and Prado, Amer. Fern J. 98 (3):139–159. 2008).

This species was cited previously by Legrand and Lombardo (1958) as being in Uruguay, and it was recorded also for Argentina by Hicken (Apuntes Hist. Nat. 1: 161. 1909), in Misiones province. The previous works that mentioned this species being in Argentina and Uruguay have not been documented with herbarium specimens. Whit this report, *S. catharinae* is officially documented for the first time for Argentina and Uruguay.

Serpocaulon catharinae has an epiphytic habit, is very common in the Uruguayan East, and it grows in association with *Butia capitata* Becc. palm groves. This fern has been collected in almost the totality of the state (''department'') of Rocha, and in the southern portion of Treinta y Tres and eastern Maldonado. Surely it is also in the state of Cerro Largo in the zone of Butia (Becc.) Becc. palm grove. A specimen of this species was found on an island in the mouth of the Santa Lucia River, in the limit of Montevideo with San José, on a specimen of *Myoporum laetum* G. Forst.; this would be the most western distribution in Uruguay. In Argentina this species has been documented only for the locality mentioned below.

SPECIMENS STUDIED: Argentina, Dpto. San Pedro, Parque Provincial Moconá, 16-XII-1992, *Seijo et al. 197* (CTES). Uruguay, Dpto. Rocha, Arroyo Zanja Honda. 20-II-2005, *Dematteis & Schinini 1656* (CTES).

Serpocaulon latipes (Langsd. & Fisch.) A.R.Sm., Taxon 55(4):928. 2006.

Polypodium latipes Langsd. & Fisch., Pl. Voy. Russes Monde 10, t. 10. 1810.

DESCRIPTIONS AND ICONOGRAPHIES: Hensen, 1990:299–301, fig. 14–16, 35, 54, 69; Labiak & Prado, 1998:53, fig. 7, Q; Sehnem, Polipodiáceae, 103–105, f. 32 (*op. cit.*), (all sub *P. latipes*).

DISTRIBUTION AND COMMENTARIES: This species occurs in southern Brazil, Paraguay, Bolivia, and northeastern Argentina (de la Sota, Opera Lilloana V:151–162, 1960). *Serpocaulon latipes* grows as a terrestrial, inside forests. This taxon usually is confused with *S. catharinae*, but as noted by Labiak and Prado (1998) and Hensen (1990), *Serpocaulon latipes* has fronds that are distantly spaced, whereas *S. catharinae* has closely-spaced petioles. Another character that we have found useful to differentiate both species is the silver-colored rhizome of *S. catharinae* that, when the scales fall off, leave exposed in certain places the pruinose layer which is deposited beneath them. This last feature was also mentioned by Labiak and Prado (2008) to differentiate both species.

With this new record *Serpocaulon* is represented by two species in Uruguay.

SPECIMEN STUDIED: Uruguay, Dpto. Rocha, Laguna Negra, Reserva de Don Bosco, S34°5′7″, W 53°45′20″, *Bonifacino et al. 2170* (CTES).

Financial support was provided by the Agencia Nacional de Promoción Científica, Tecnológica y de Innovación (ANPCyT-UNNE, PICTO 00096) and by the Secretaría General de Ciencia y Técnica de la Universidad Nacional del Nordeste (PI 038/06). Thanks to Melanie Link-Pérez for critical reading of the manuscript—ESTEBAN I. MEZA TORRES, Instituto de Botánica del Nordeste, Sargento Cabral 2131, C.C. 2009, Corrientes, Argentina, E-mail: mezatorresii@yahoo.com.ar, HÉCTOR A. KELLER, Instituto de Botánica del Nordeste, Sargento Cabral 2131, C.C. 2009, Corrientes, Argentina, and CARLOS A. BRUSSA, Museo y Jardín Botánico Prof. Atilio Lombardo, Av.19 de Abril 1181, C.P. 11700, Montevideo, Uruguay, E-mail: cabrussa@adinet.com.uy.

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AMERICAN FERN JOURNAL

Volume 100

Number 4

October–December 2010

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The "American Fern Journal" (ISSN 0002-8444) is an illustrated quarterly devoted to the general study of ferns. It is owned by the American Fern Society, and published at The American Fern Society, % Missouri Botanical Garden, P. O. Box 299, St. Louis, MO 63166-0299. Periodicals postage paid at St. Louis, MO, and additional entry.

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American Fern Journal 100(4):189–194 (2010)

A New Combination in *Pleopeltis* and Some Nomenclatural Notes Related to Illustrations Validating Fern Names

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ABSTRACT.—We present the new combination *Pleopeltis minima* (Bory) J. Prado & R. Y. Hirai and we selected a lectotype for *Marginaria minima* described by Bory, based on material from southern Brazil. This taxon has a wide range in the southern part of South America and it has been recognized in the rank of species and/or variety by several previous authors, but its correct name has been misleading. Recently it has been treated as a member of *Pleopeltis*, but with a wrong epithet. The nomenclatural mistake involving this species arose because there are different interpretations of some nomenclatural rules defined in the IBCN. A similar misunderstanding of the same rule is also discussed for a species in *Lindsaea*.

KEY WORDS.—*Pleopeltis*, *Lindsaea*, nomenclature

During our participation as reviewer or author of an annotated checklist of the ferns and lycophytes from *Cono Sur* (by the first author; Ponce *et al.*, 2008) and from Brazil by both authors (Labiak and Hirai, 2010), we discovered problems involving a name for a species in Polypodiaceae. These problems are related to the correct understanding of the Articles 42.3 and 42.4 of the current Code (McNeill *et al.*, 2006) regarding illustration with analyses validating names at species and infraspecies levels, and the selection of a correct basionym for a new combination.

The provisions of these Articles in the current Code (McNeill *et al.*, 2006) are: "***42.3.*** *Prior to 1 January 1908 an illustration with analysis, or for non-vascular plants a single figure showing details aiding identification, is acceptable, for the purpose of this Article, in place of a written description or diagnosis.* ***42.4.*** *For the purpose of Art. 42, an analysis is a figure or group of figures, commonly separate from the main illustration of the plant (though usually on the same page or plate), showing details aiding identification, with or without a separate caption.*"

In other words, a published illustration can be used to validate a name of a vascular plant published before 1908, but this illustration needs to show adequate details to match the requirements of both Articles to therefore be considered acceptable. This provision has been in the Code since the Congress of Vienna in 1905 (Morton, 1967), but the Sydney Code (Voss *et al.*, 1983) introduced a clarification that a caption is necessary in order to consider an illustration validly published; this caption can be separate or together with the illustration.

At least two examples of misinterpretation of these rules can be taken from monographs and recently published floras for neotropical ferns. There are probably other similar examples, but we present just two of them, as follows.

The first example is related to a polypod fern belonging to the *Polypodium polypodioides* complex, revised by Weatherby (1939). These are epiphytic plants with laminar surfaces overlain by scales. In the southern part of South America, a focus of our studies, at least six names have been applied to a species from this complex, in different genera and ranks: *Marginaria minima* Bory, *Polypodium minimum* (Bory) Herter, *Polypodium polypodioides* (L.) Watt var. *minimum* (Bory) Kuhlm. & Kuhn, *Goniophlebium incanum* (Sw.) J. Sm. var. *minus* Fée (as "minor"), *Polypodium polypodioides* (L.) Watt var. *minus* (Fée) Weath., and *Polypodium squalidum* Vell.

Polypodium squalidum was combined in *Pleopeltis* by Sota (2003) in order to accommodate this species in the new sense of *Pleopeltis* proposed by Andrews and Windham (1993). Sota (2003) based his decision on information presented by Burkart (1963), who adopted the name published by Vellozo (1831 [1827], 1881). Three questions arise from this nomenclatural act: 1) is Vellozo's name validly published?; 2) is *Polypodium squalidum* the oldest name for this taxon?; and 3) if not, is there another name available to be combined in *Pleopeltis*?

As discussed by Weatherby (1939) and Morton (1967), names published by Vellozo (1831 [1827]) in *Florae Fluminensis Icones* in 1827 (actually, as pointed out by Carauta (1973), the effective date of publication of *Florae Fluminensis Icones*, vol. 11 is 1831) were not validly published because they were accompanied only by illustrations that did not show essential characters. Thus, not all requirements of the Code for valid publication were fulfilled. We agree. However, when the descriptions for the Vellozo's species were published in 1881, these names became validly published, but the date of the validity of the names is 1881, not 1831 [1827].

We also conclude, as did Burkart (1963), that *Polypodium squalidum* is not the oldest name for this species, and that *Marginaria minima* Bory is an older basyonym available for this species in *Pleopeltis*.

Burkart (1963) noted that the epithet "*minimum*" was pre-occupied in *Polypodium*, so he adopted the next available name, *Polypodium squalidum* Vell., as the name for this species. His conclusion was based on the fact that the Vellozo's name was validly published in 1831 [1827]. In this point we believe he erred, because he considered both figures diagnostics for the taxon.

Weatherby (1939) pointed out that the attachment of the scales on the lamina (appressed or somewhat spreading), scale margins, and scale position on the laminar surfaces are the essential characters for distinguishing this taxon from related ones (*Polypodium polypodioides* var. *michauxianum* Weath.; *P. polypodioides* var. *aciculare* Weath.). Also, the venation pattern (free) in *P. polypodioides* var. *minus* differs from that in *Polypodium ecklonii* Kunze, which has anastomosing veins. Unfortunately, the details of veins and scales are not visible on the figure published by Vellozo.

Despite the fact that there is an older name for the *Polypodium squalidum*, many recent authors have used the combination published by Sota (2003) in *Pleopeltis*, e.g., Pensiero and Gutiérrez (2005), Schwartsburd and Labiak (2007), Labiak *et al.* (2008), Prado and Labiak (2009), Assis and Labiak (2009), and Salino and Almeida (2010).

For the species heretofore known as *Pleopeltis squalida* (Vell.) de la Sota, we propose a new combination and choose a lectotype as follows:

New combination:

Pleopeltis minima (Bory) J. Prado & R. Y. Hirai, ***comb. nov.*** *Marginaria minima* Bory, Dict. Class. Hist. Nat. 10: 177. 1826. *Goniophlebium incanum* (Sw.) J. Sm. var. *minus* Fée, Crypt. Vasc. Brésil 1: 107. 1869, as "minor". *Polypodium minimum* (Bory) Herter, Anales Mus. Nac. Montevideo, ser. 2, 1: 368, tab. 28. 1925, *nom. illeg.*, non Aublet (1775), nec Brackridge (1854), T. Moore (1857), Baker (1879). *Polypodium polypodioides* (L.) Watt var. *minus* (Fée) Weath., Contr. Gray Herb. 124: 31. 1939. *Polypodium polypodioides* (L.) Watt var. *minimum* (Bory) Kuhlm. & Kuhn, Fl. Distr. Ibiti: 22. 1948. Lectotype here chosen: "parties témprées du Brésil" Santa Catarina, *Crypt. Voy. de la Coquille 61* (P00632926!). **Fig. 1.**

Polypodium squalidum Vell., Fl. Flumin. Icon. 11: t. 76. 1831 [1827] (*nomen nudum*), Arch. Mus. Nac. Rio de Janeiro 5: 449. 1881. *Pleopeltis squalida* (Vell.) de la Sota, Hickenia 3(46): 196. 2003. Lectotype chosen by Burkart (1963): Fl. Flumin. Icon. 11: tab. 76.

Polypodium microlepis Fée, Gen. fil.: 238. 1852, *ex descr.* Type: America Australi, Herb. Mougeotiano, *Pamplin 38* (P, not seen).

Distribution and habitat.—This species grows epiphytically or terrestrially in forests; Bolivia, Argentina, Brazil, Paraguay, and Uruguay; 0–1800 m.

The sheet containing the lectotype here selected has two collections by Coquille, one with the barcode P00632925 (*Coquille 60*) and the other P00632926 (*Coquille 61*). Above the specimen of Coquille *60* there is an annotation pointing out that it was collected in Santa Catarina State in 1827, one year after the publication of the name by Bory (1826).

For an additional list of synonyms for this taxon, see Weatherby (1939, p. 31, under *Polypodium polypodioides* var. *minus* (Fée) Weath.).

A second example of the same problem, publication of a species name by using an inadequate illustration, was documented by Morton (1967), who discussed the adoption of the name *Lindsaea klotzschiana* Moritz ex Ettingsh. by Kramer (1957). According to Morton (1967), *Lindsaea feei* C. Chr. is the correct name for the species, because *L. klotzschiana* was not validly published either by Moritz (1854, apud Morton 1967) or by Ettingshausen (1865, apud Morton 1967). In Morton's (1967) opinion, Moritz (1854) published a *nomen nudum* because Ettingshausen (1865) published only

FIG. 1. Photograph of the lectotype of *Marginaria minima* Bory (©MNHN, Paris Herbarium, 2010).

one small illustration showing details of the venation of a pinna. Morton concluded that this was insufficient to meet the provisions for valid publication in the Code (at that time, the Edinburgh Code, Lanjouw *et al.*, 1966). Even though Morton (1967) called attention to this point, unfortunately, most recent floras have adopted the name *Lindsaea klotzschiana* instead of *L. feei*, for example, Stolze (1981), Murillo-Pulido and Harker-Useche (1990), Moran (1995), Smith (1995), Mickel and Smith (2004), and Gómez and Arbeláez (2009). So, to solve this nomenclatural problem we suggest the following citation for both species name:

Lindsaea feei C. Chr., Index Filic. 393. 1906, ***nom. nov.*** for *L. elegans* Fée, Gen. Filic. (Mém. Foug. 5) 106. 1852, *nom. illeg.*, non Hooker (1837). *Lindsaea klotzschiana* Moritz ex Ettingsh., Farnkr. Jetztw. 212, pl. 145, f. 12. 1865(1864), *nom. nud.* Lectotype chosen by Kramer (1957): Venezuela, Tovar, *Moritz 238* (P00538895!, duplicates B!, P!, W not seen).

To avoid future nomenclatural problems related to the correct interpretation of Art. 42.4 we proposed a new wording for this Article, plus a new explanatory Note and one example (Prado & Hirai 2010).

Acknowledgments

We thank The Muséum National D'Histoire Naturelle (MNHN) and Dr. Germinal Rouhan (P) for providing the photo of the type specimen of *Marginaria minima*, Dr. Alan R. Smith (UC) and Dr. Michael Sundue (NY) for their comments on the manuscript, and one anonymous reviewer for suggestions to improve the text.

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American Fern Journal 100(4):195–206 (2010)

Adiantum camptorachis (Pteridaceae), a New Species from South America with Notes on the Taxonomy of Related Species from the Southern Cone and Bolivia

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ABSTRACT.—We describe *Adiantum camptorachis* from the Tucumano-Boliviano forests of southern Bolivia and northwestern and central Argentina. This species was previously confused with *A. glanduliferum*, which is found on the Pacific side of Andes from southern Peru to central Chile. *Adiantum camptorachis* differs by having rhizome scales with distally ciliate margins, erose pinna margins, and by having a flexuous rachis. We also provide a detailed description for *A. rufopunctatum*, which had previously been treated as a synonym of *A. raddianum* by several authors. A key to similar South American species is presented. It includes all species with decompound non-conform laminae and orbicular to reniform or lunate sori in the Southern Cone, including Argentina Chile, Bolivia, Brazil, Paraguay, and Uruguay.

KEY WORDS.—*Adiantum*, taxonomy, new species

Taxonomic and nomenclatural confusion has surrounded species of maidenhair ferns (*Adiantum*) with decompound, non-conform laminae and orbicular to reniform indusia. In the central and southern Andean regions, our recent focus, this confusion largely concerns three species, *Adiantum chilense* Kaulf., *A. poiretii* Wikstr., and *A. raddianum* C. Presl. Problems noted by previous authors include the morphological distinction between *A. chilense* and *A. poiretii* (de la Sota, 1977; Tryon and Stolze, 1989), and the correct name for the latter (Pichi-Sermolli, 1957; Tryon and Stolze, 1989). Our research (Sundue, 2011; Prado, unpublished data) suggests that all three names have often been too broadly applied to also include narrowly restricted taxa in the region (Tryon, 1964; de la Sota, 1977; Tryon and Stolze, 1989; Rodríguez, 1995; Giudice, 1999). Our current contribution, which grew out of recent efforts to resolve the taxonomy and nomenclature of Bolivian species, serves to redefine each of these taxa by the description of one new species, the resurrection of one old species, and by providing a key to identification of similar species in the Southern Cone of South America, an area which we treat here to include Argentina, Chile, Bolivia, Brazil, Paraguay, and Uruguay.

Although the relationships among the non-conform species of *Adiantum* with rounded or fan-shaped ultimate segments are still unclear, they do not all fall within the same clade (Huiet and Smith, 2004; Huiet *et al.*, in prep.), on the

basis of coding and non-coding chloroplast markers (*rbcL*, *rps4*, *rps4-trnS* spacer). Many species in the Southern Cone, including *A. raddianum*, *A. poiretii*, *A. lorentzii* Hieron., and *A. digitatum* Hook., as well as most others still unsampled for molecular markers in the key below, appear to belong to what is here called the *raddianum* clade, comprising mostly Neotropical (especially South American, a few African and Australasian) species that lack silica bodies between the veins (silica bodies are often referred to as "idioblasts" in fern taxonomy, e.g., Wagner, 1978; but see Sundue, 2009), have veins running to sinuses, non-abscising segments, and color of the segment stalks passing into the base of the segments. However, *Adiantum capillus-veneris* L., with veins running to the tips of teeth, belongs to a different, only distantly related clade. The circumscription of the *A. raddianum* clade, as inferred from molecular markers, corresponds only in part to Groups 1 and 2 (*A. capillus-veneris* Group, *A. patens* Group) of Tryon and Tryon (1982: 321–322).

The key provided includes all native species that are commonly confused with *A. chilense*, *A. poiretii*, and *A. raddianum*, or that appear to be closely related. Species included were compiled from Foster (1958), Giudice (1999), Labiak and Prado (2007), Ponce (1996), Ponce *et al.* (2008), Prado (unpublished data), Rodríguez (1995), Sundue (in press), de la Sota (1977), and from the collections at LPB, NY, SP, UC, and USZ. These species have 2–4-pinnate decompound non-conform laminae. Sori in this group range from orbicular to reniform or lunate, but are not linear. Ultimate segments are orbicular, obovate, rhomboid, or flabellate, and do not have silica bodies between the veins. Species with larger, trapeziform, ultimate segments such as *A. mathewsianum* Hook., *A. pentadactylon* Langsd. & Fisch., and *A. trapeziforme* L., are excluded.

Adiantum camptorachis Sundue, J. Prado & A. R. Sm., ***sp. nov.*** TYPE.—Bolivia. Depto. Tarija, Prov. Arce, Municipio Padcaya, Reserva Nacional de Flora y Fauna Tariquía, bajando de la cima del Cerro Lorayoj al Abra del Lorayoj, 21°54′18″S, 64°54′14.1″W, 3200 m, 3 Aug 2005, *M. Serrano, J. Villalobos & A. Lliully 6148* (holotype NY!; isotypes HSB, MO!). **Fig. 1, Fig. 2A, B.**

Ex affinitate *A. glandulifero* Link, squamis rhizomatum ciliatis (vs. squamis integerrimis in *A. glandulifero*), segmentis marginibus erosis (vs. segmentis denticulatis in *A. glandulifero*) et rhachibus flexuosis (vs. rhachibus rectis in *A. glandulifero*) differt.

Etymology.—Campto-(Greek: *kamptos*, bent) + rachis; referring to the flexuous or zig-zag rachis of the leaf.

Plants terrestrial; rhizomes long-creeping, 1.0–2.5 mm wide, castaneous, forming branch buds, with internodes 1–3 cm long, these sparsely scaly, the nodes and rhizome apices densely scaly, scales 1.5–2.5 × 0.3–1.0 mm, lanceolate, castaneous, shiny, basifixed, the apices acute, margins distally

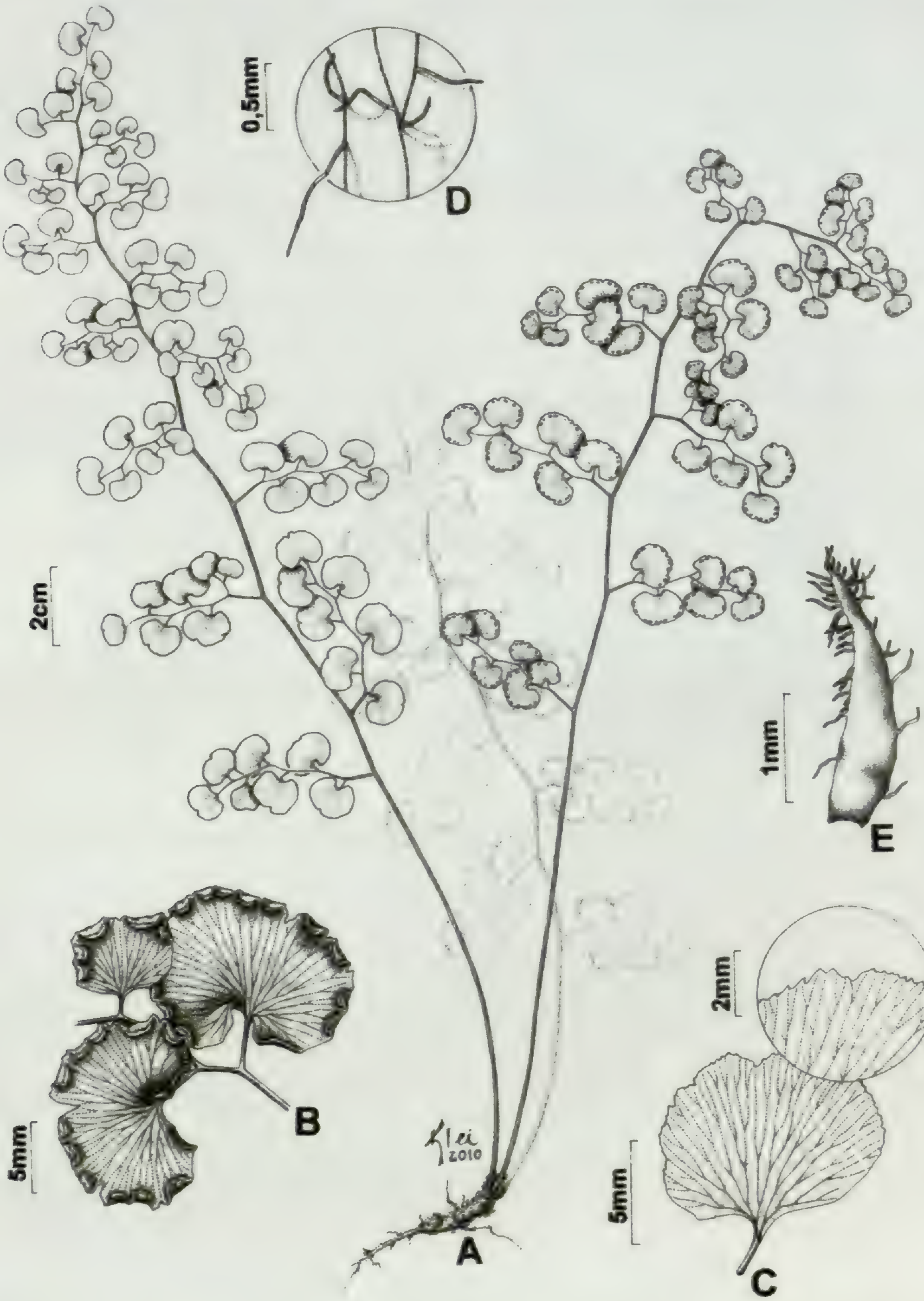

FIG. 1. A–E. *Adiantum camptorachis*. A. Habit. B. Abaxial view of fertile pinnules. C. Abaxial view of the sterile pinnule and a detail of its margin. D. Articulate hairs on pinnule veins abaxially. E. Rhizome scale (A–D, from *Ibisch & Rojas 325*, LPB; E, from *Hennipman & Rödl-Linder 8093*, LPB).

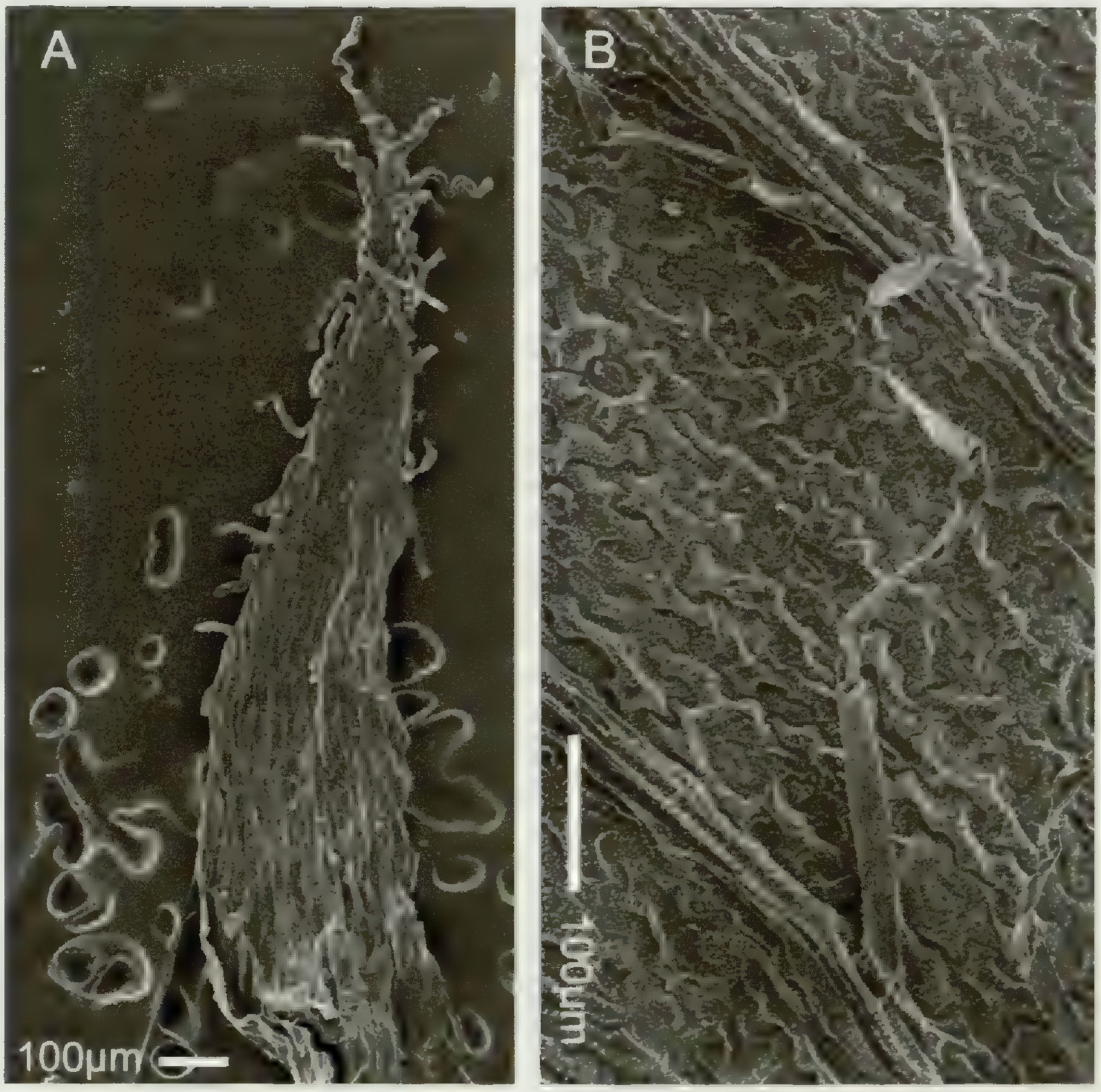

FIG. 2. *Adiantum camptorachis*. A. Rhizome scale. B. detail of a hair on the vein from abaxial laminar surface (both from *Serrano et al. 6148*, NY).

ciliate, the cilia 0.1 mm long, tortuous, spreading, the same color as the body of the scale (Fig. 1E; 2A); leaves 30–75 × 8–20 cm, closely spaced or to 3 cm apart; petioles 10–20 cm × 1–2 mm, castaneous, lustrous, very slightly glaucous or not, glabrous or with a few scales at the base like those of the rhizomes; rachises flexuous, glabrous (Fig. 1A); laminae without apparent silica bodies between veins on both laminar surfaces, oblong to lanceolate, 2–3-pinnate, apices gradually reduced and without a conform terminal pinna, with 7–10 pinna-pairs, the ultimate segments orbicular, petiolulate, with the color of the petiolules passing into the veins at the base of the segments, 0.7–1.5 × 1.5–2.0 cm, the bases truncate to subcordate, the apices shallowly lobed, the margins denticulate and erose (Fig. 1C), the abaxial laminar surfaces slightly glaucous, sparsely villous along the veins, the hairs 0.5–1.0 mm long, 5–8-celled, whitish, slightly tortuous, spreading (Fig. 1D; 2B), the adaxial laminar surfaces glabrous; veins flabellate, ending in sinuses between marginal teeth in sterile segments; sori 1.0–3.0 mm wide, oblong to reniform, whitish, glabrous; spores brown.

OTHER SPECIMENS EXAMINED.—ARGENTINA. Prov. Córdoba: Sierra de Achala, Cuesta del Tránsito, 6–13 Jan 1895, *Kurtz 8353* (NY). BOLIVIA. Depto. Cochabamba: "bei

Cochabamba", 3000 m, 26 March 1892, *Kuntze s.n.* (NY, 2 sheets); Prov. Arque, Comunidad Huancani, 3600 m, 11 Aug 1991, *Ibisch & Rojas 325* (LPB); Prov. Ayopaya, above Independencia, 3400 m, 5 May 1990, *E. Hennipman & Rödl-Linder 8093* (LPB); Prov. Ayopaya, 2 km de Casay Vinto–Choro, 16°32′S, 66°38′W, 3350 m, 3 May 1997, *Kessler et al. 9240* (LPB not seen; UC). Depto. Cochabamba or Depto. Santa Cruz: Sierra de Santa Cruz, 3000 m, May 1892, *Kuntze s.n.* (NY, 2 sheets).

Distribution and habitat.—Restricted to the Tucumano-Boliviano forests of southern Bolivia and northwestern and central Argentina, 3000–3600 m.

Discussion.—*Adiantum camptorachis* was previously confused with *A. glanduliferum* Link, a species sometimes treated as a synonym of *A. chilense* var. *hirsutum* (Hook. & Grev.) R. M. Tryon (see Rodríguez, 1995) and found on the Pacific side of the Andes in southern Peru and northern and central Chile, from pre-cordilleran foothills to 1550 m. Specimens pertaining to *Adiantum camptorachis* were treated as *A. thalictroides* var. *hirsutum* (Hook. & Grev.) de la Sota by de la Sota (1977) in the Flora de la Provincia de Jujuy and with *A. glanduliferum* in synonymy, but this was in error. The two species have similar laminar dissection, segment shape, and abaxial laminar indument of spreading whitish hairs, and judging by these characters alone, the similarity between them is striking. However, *A. glanduliferum* (Fig. 3A, B), and all other Chilean species with decompound laminae, differ by having rhizome scales with entire margins. *Adiantum camptorachis* further differs from these species by having flexuous rachises, and erose segment margins. By comparison, *A. glanduliferum* has a relatively straight rachis, and denticulate segment margins. Rodríguez (1995) reported *A. glanduliferum* as being pubescent on both sides of the lamina, which we have seen in some specimens (Chile, near Valparaíso, 16 Sep 1914, *Rose & Rose 19125*, NY) but not in others (Chile, *Bertero s.n.*, NY; Chile, Valparaiso, 1837, *Gaudichaud s.n.*, NY; Chile, Prov. Valparaíso, Casablanca, Cuesta Lo Orozco, 400–500 m, 4 Nov 1956, *Kausel 4281*, NY), which are pubescent upon the abaxial side of the lamina only. Specimens that are pubescent on both sides of the laminae are also pubescent between the veins and upon the segment margins, whereas in specimens that are pubescent only on the abaxial side of the lamina, the pubescence is restricted to the veins. The significance of these differences should be investigated further.

We also believe that the numerous types cited by de la Sota (1977) for *Adiantum thalictroides* var. *hirsutum*, and by Rodríguez (1995) for *A. chilense* var. *hirsutum*, apply to Chilean species and not to *A. camptorachis*, judging by the provenance of the type collections (Looser, 1940) and by their having rhizome scales with entire margins.

Several other species of *Adiantum* with decompound laminae and orbicular to reniform indusia that occur in northern Argentina and Bolivia could also be confused with *A. camptorachis*, including *A. lorentzii* Hieron., *A. orbignyanum* Kuhn, *A. pseudotinctum* Hieron., *A. raddianum*, and *A. rufopunctatum* Mett. ex Kuhn. However, all of these species differ by rhizome scales with

FIG. 3. A–B. *Adiantum rufopunctatum*. A. Habit. B. Abaxial view of fertile pinnule (from *Beck 8726*, LPB). C–D. *A. poiretii*. C. Habit. D. Abaxial view of fertile pinnules (from *Beck & Paniagua 27125*, LPB).

entire margins. Furthermore, all of them except *A. orbignyanum* and *A. pseudotinctum* differ by having short-creeping rhizomes.

We suspect that *Adiantum camptorachis* is closely related to *A. poiretii* (Fig. 3C, D), which is distributed from Mexico and the West Indies south to Argentina, Tristan da Cunha, southern Brazil, central and southern Africa, Madagascar, the Mascarenes, and India. Both species share characteristic castaneous rhizome scales with distally ciliate margins, as well as having long-creeping rhizomes. From *A. poiretii*, the new species can be distinguished by its flexuose rachises, erose segment margins, and by the pubescence of the laminae abaxially.

Adiantum poiretii is variable throughout its range, particularly in the degree to which the rhizome scales are ciliate, and whether or not the sori are yellow-farinose. This variation does not include characters that overlap with those of *A. camptorachis*, nonetheless we acknowledge the need for further study of *A. poiretii* throughout its range. Pichi-Sermolli (1957) considered *A. poiretti* to be endemic to Tristan da Cunha, and *A. thalictroides* Willd. ex Schltdl. to be the correct name for plants of this complex from elsewhere. Most recent authors, however, have disagreed (R. M. Tryon, 1964; A. F. Tryon, 1966; Tryon and Stolze, 1989; Moran, Zimmer and Jermy, 1995; Rodríguez, 1995; Mickel and Smith, 2004) and applied the name *A. poiretii* broadly. We have not studied the type of this species (*du Petit-Thouars 1427*, P), and believe that more comprehensive studies are needed to resolve the circumscription of the species. Manton and Vida (1968) reported a chromosome number of $n = 114$ for plants of *A. poiretii* from Tristan da Cunha, twice the number counted from continental Africa. Christensen (1940), in his flora of Tristan da Cunha, described plants there as having rhizome scales with entire margins, whereas material from Africa, Madagascar, and the Americas has scales with distally denticulate margins. However, illustrations of rhizome scales of plants from the nearby Gough Island (based on *Hänel s.n.*, NBG, kindly made available to us by Koos Roux) show that those scales are not entire, but in fact sparsely ciliate.

Adiantum rufopunctatum Mett. ex Kuhn, Jahrb. Königl. Bot. Gart. Berlin 1: 350. 1881. TYPE.—Bolivia. Yungas, [without precise date], *A. C. V. D. d'Orbigny 165* (lectotype B n.v., chosen by R. M. Tryon (1964), BGBM virtual herbarium image Id: 262880 (Röpert, 2000), photo GH, US; isotypes P n.v. MNHN image barcode: P00608520, F! fragment, MO n.v. fragment, NY! fragment). **Figs. 3A–B, 4.**

Adiantum boliviense H. Christ & Rosenst., Repert. Spec. Nov. Regni Veg. 5: 230. 1908. TYPE.—Bolivia. Prov. La Paz, Sud Yungas, Yanacachi prope La Florida, 16° S, 1600 m, 21 Nov 1906, *O. Buchtien 459* (Lectotype S n.v., designated by R. M. Tryon (1964); isolectotypes P n.v. MNHN image online barcode: P00608521, UC!, US! barcode: 00142178).

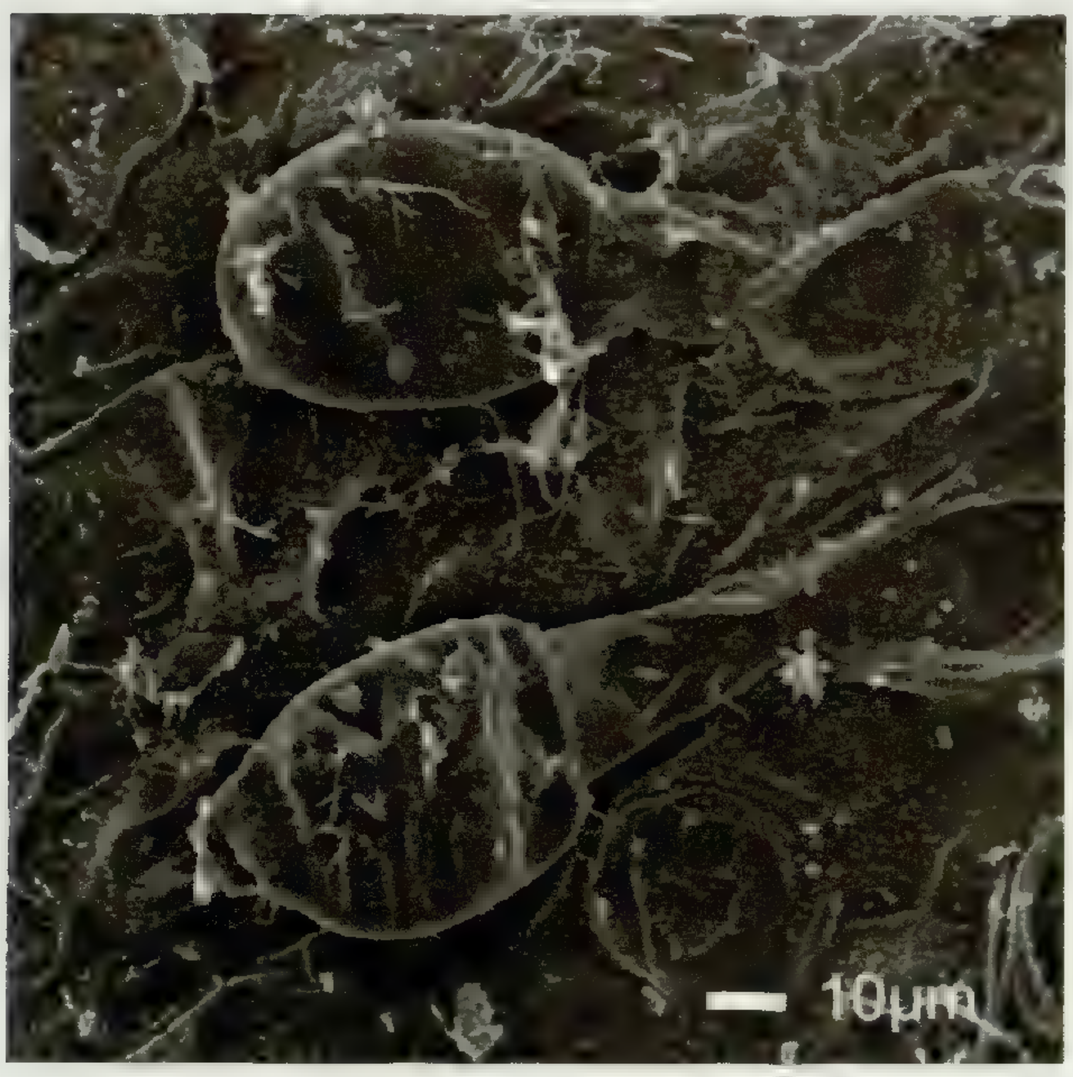

FIG. 4. *Adiantum rufopunctatum*. Detail of a glandular hair 2-celled between veins from abaxial laminar surface (from *Bang 2964*, NY).

Adiantum decorum var. *quadripinnata* Rosenst., Meded. Rijks-Herb. 19: 8. 1913. TYPE.—BOLIVIA. [Depto. Cochabamba or Depto. Santa Cruz] Yungas de San Mateo, 1500 m, [without precise date], *T. C. J. Herzog 1995* (Lectotype **here designated** US! barcode: 00142180, isolectotype S?).

Plants terrestrial; rhizomes short-creeping, ca. 4 mm wide, the rhizome apices densely scaly, the scales 2.0–3.5 × 0.3–0.6 mm, narrowly lanceolate, light brown to castaneous, shiny, basifixed, the apices long-attenuate, margins entire; leaves 30–50 × 15–20 cm, caespitose; petioles 10–20 cm × 1–2 mm, castaneous, lustrous, the bases very slightly glaucous, glabrous or with a few scales at the base like those of the rhizomes; rachises essentially straight, not conspicuously flexuous; laminae without apparent silica bodies between veins on both laminar surfaces, ovate to deltate, 3–4-pinnate, with 5–10 pinnae, the apices gradually reduced and without a conform terminal pinna, the ultimate segments orbicular, petiolulate, with the color of the petiolules passing into the veins at the base of the segments, 0.7–1.5 × 1.5–2.0 cm, the bases cuneate, apices rounded to shallowly lobed, the abaxial laminar surfaces evenly glandular, the glands 2-celled, reddish, ca. 0.04 mm wide (Fig. 3B, 4), otherwise glabrous, the adaxial laminar surfaces glabrous; veins flabellate, ending in sinuses of sterile segments; sori 1.0–3.0 mm wide, orbicular, whitish, glabrous; spores brown.

SELECTED SPECIMENS EXAMINED.—PERU. Depto. Cuzco: Prov. La Convención, approx. 150 km de Cuzco en la vía carretera Cuzco-Quillabamba, 12°57′S, 72°40′W, 1100–1300 m, 14 Nov 1987, *Núñez 8594* (NY); Prov. La Convención, abajo de Echarate en el Alto Urubamba, 12°47′S, 72°40′W, 670 m, 3 Feb 1989, *Núñez et al. 10154* (NY); Prov. La Convención, western affluent of Vilcanota, valley of Sambray, 1200 m, 10 May 1936, *Mexia 8033* (NY, UC); Prov. La

Convención, Dist. Maranura, Santa María, 13°00′17″ S, 72°33′02″W, 1207 m, 27 Mar 2004, *Huamantupa & Huamantupa 4525* (MO n.v., UC); Quillabamba, 1200 m, 21 Feb 1954, *Coronado 129* (UC). Depto. Junín: Prov. Tarma, near Vitoc, along San Ramón – Monobamba rd. 1190 m, 22 May 1978, *Skog et al. 5010* (NY); Huacapistana, 1812 m, 30 Oct 1954, *Coronado 276* (UC). BOLIVIA. Depto. La Paz: Prov. Bautista Saavedra, Area Natural de Menejo Integrado Apolobamba, Camata, puente sobre el Río Chatazani–Camata, 15°13′39″S, 68°45′40″W, 1450 m, 19 Apr 2005, *Miranda et al. 731* (MO n.v., UC); Prov. Bautista Saavedra, 10 km de Camata hacia Apolo, 15°13′S, 68°41′W, 1300 m, 25 Jun 1997, *Kessler et al. 10332* (LPB n.v., UC); Prov. Caranavi, camino Caranavi-Palos Blancos, 15°48′S, 67°28′W, 1280 m, 27 Sep 2001, *Jiménez 658* (LPB n.v., UC); Prov. Franz Tamayo, San Juan, 3800 ft, 1901–1902, *Williams 1311* (NY, UC); Bolivia: Nor Yungas, Coripata 12 km hacia el S, vía Puente Villa, Quebrada húmeda con arroyo 16°22′S, 67°36′W, 1600 m, 31 Dec 1983, *Beck 8726* (LPB, SP); Prov. Nor Yungas, Milluguaya, 1300 m, Jul 1917, *Buchtien 5036* (NY, UC); Prov. Nor Yungas, 2.6 km below Yolosa, 1200 m, 22 Jan 1988, *Grifo & Solomon 741* (NY, UC); Prov. Nor Yungas, valley of Río Yolosa, near Yolosa, ca. 1500 m, 5 Dec 1935, *Mexia 7800* (UC); Prov. Nor Yungas, fin del camino del Inca, puente de Huarinilla, 450 m, 27 Mar 1992, *Schmit 459* (UC); Prov. Sud Yungas, basin of Río Bopi, San Bartolomé, near Calisaya, 750–900 m, 1–22 July 1939, *Krukoff 10043* (NY); Prov. Sud Yungas, Pitiguaya, valley of Unduavi River, 5800 ft, 7–15 May 1926, *Tate 762* (NY). Depto. Santa Cruz: Prov. Florida, comunidad de Bella Vista, 18°11′57.75″S, 63°41′21.46″W, 1000–1100 m, 4 Aug 2006, *Villarroel et al. 517* (NY). Depto. Chuquisaca: Prov. Luis Calvo, bajando de la cima de la Serranía del Iñao, 19°32′28″S, 63 55′03″W, 1820 m, 8 Nov 2001, *Huaylla 146* (NY). Without precise locality, *Bang 2064* (NY, UC).

Distribution and habitat.—Humid montane forests from central Peru to southern Bolivia, (670–)1100–1500(–1820) m.

Adiantum rufopunctatum was treated as a synonym of *A. raddianum* C. Presl by Tryon (1964), a species with a much larger range and known from the Antilles, Mexico, Central America, Colombia, Venezuela, Ecuador, Peru, Bolivia, Brazil, Paraguay, Uruguay, and Argentina from 200–1800(3000) m. However, *A. rufopunctatum* can be readily distinguished from *A. raddianum* by having reddish glands, 2-celled, ca. 0.04 mm wide on the abaxial laminar surfaces (Figs. 3B, 4).

The lamina architecture of *Adiantum rufopunctatum* will also often contrast strongly with that of *A. raddianum*, but some plants overlap in these characters. *Adiantum rufopunctatum* is characterized by laminae that are 3–4-pinnate. The basal pinnae have well developed basiscopic pinnules, to 4.0 cm long, and all of the pinnae tend to have well developed acroscopic pinnules that are (1.5–)4.0–5.5(–6.5) cm long. The ultimate segments of *A. rufopunctatum* are 3.0–14.0 × 1.5–13.0 mm long, oblanceolate to obovate or flabellate, with cuneate bases, and rounded to lobed apices (Fig. 3B). By contrast, the laminae of *A. raddianum* are 2–3(4)-pinnate, and the pinnae are oblong to lanceolate, bearing acroscopic pinnules that are 1.3–2.8 cm long. The ultimate segments are (4.0–)8.0–19.0 × (3.0–)4.0–18.0(–22.0) mm, and are

oblong to obdeltate, the bases are obtuse, square, or cuneate, and apices are lobed and often incised.

Key to the Decompound (2–4-pinnate) Non-conform Species of *Adiantum* in the Southern Cone and Bolivia

1. Ultimate segments deeply lobed, often more than half the length of the segment; fronds subscandent; axes with raised aerophores at each branching point, the aerophores often pale. . . . . *A. digitatum*
1. Ultimate segments shallowly lobed, less than half the length of the segment; fronds erect, not subscandent; axes smooth, without aerophores, or the aerophores inconspicuous, not differing in color.
2. Veins of sterile laminar segments terminating in teeth of the segment margins. . . . . *A. capillus-veneris*
2. Veins of sterile laminar segments terminating at the base of sinuses between teeth of the segment margins, or the margins of sterile segments without teeth and sinuses, essentially entire.
3. Ultimate segments articulate, the articulations at the apices of the segment petiolules swollen; dark color of the segment petiolules stopping abruptly at the point of articulation and not passing onto the bases of segments. . . . . *A. orbignyanum*
3. Ultimate segments not articulate; apices of segment petiolules not swollen; dark color of segment petiolules changing gradually, often fading before, or passing into the bases of segments.
4. Sori orbicular to reniform.
5. Laminae pseudo-pedately divided, often with two ± equal rachises each bearing 1-pinnate pinnae on one side, sometimes with single central rachis; rachises with branched whitish hairs, ca. 0.1 mm long; rhizome scales golden, the margins ciliate. . . . . *A. patens*
5. Laminae pinnately divided, always with a single central rachis; rachises glabrous; rhizome scales light to dark brown, or castaneous, the margins entire to sparsely denticulate.
6. Basal pinnae subsessile, the proximal ultimate acroscopic segments of basal pinnae suborbicular, usually conspicuously overlapping the rachis; rhizome scales sparsely denticulate. . . . . *A. subvolubile*
6. Basal pinnae stipitate, the proximal ultimate segments of basal pinnae cuneate, usually not overlapping the rachis; rhizome scales entire.
7. Rhizomes long-creeping. . . . . *A. pseudotinctum*
7. Rhizomes short-creeping to erect.
8. Sori yellow-farinose; rhizomes erect or suberect . . . . . *A. lorentzii*
8. Sori not yellow-farinose; rhizomes short-creeping to suberect.
9. Rhizome scales stramineous to ferrugineous or light brown, not conspicuously lustrous . . . . . *A. sinuosum*
9. Rhizome scales castaneous, lustrous.
10. Abaxial laminar surfaces glandular, the glands 2-celled, reddish, 0.04 mm wide; laminae 3–4-pinnate; pinnae broadly lanceolate; acroscopic pinnules (1.5–)4.0–5.5(–6.5) cm long; ultimate segments 3.0–14.0 × 1.5–13.0 mm . . . . . *A. rufopunctatum*
10. Abaxial lamina surfaces without glands; laminae 2–3(4)-pinnate; pinnae oblong to narrowly lanceolate; acroscopic pinnules 1.3–2.8 cm long; ultimate segments (4.0–)8.0–19.0 × (3.0–)4.0–18.0 (–22.0) mm . . . . . *A. raddianum*
4. Sori oblong to lunate.
11. Rhizome scale margins ciliate throughout, or at least distally.
12. Abaxial laminar surfaces glabrous; rachises essentially straight, not conspicuously flexuous; sori yellow-farinose or not. . . . . *A. poiretii*

12. Abaxial laminar surfaces villous, the hairs 0.5–1.0 mm long, 5–8-celled, whitish, slightly tortuous, spreading; rachises flexuous; sori not yellow-farinose. . . . . ***A. camptorachis***

11. Rhizome scale margins entire.

13. Laminar surfaces entirely glabrous, lacking hairs, glands, and farina, or if yellow farina present then confined to the sori.

14. Axes with septate hairs and narrow hairlike scales, the hairs and scales to 2.0 mm long . . . . . *A. excisum*

14. Axes glabrous. . . . . *A. chilense*

13. Laminae pubescent, farinose, or glandular.

15. Laminae yellow-farinose or with sessile yellow glands ca. 0.1 mm wide; laminae lacking hairs . . . . . *A. sulphureum*

15. Laminae non-farinose or white-farinose, sessile glands absent; laminae pubescent.

16. White farina present at apices of the laminar hairs . . . . . *A. scabrum*

16. Farina absent.

17. Axes and laminae pubescent, the hairs to ca. 1.5 mm long. *A. gertrudis*

17. Axes glabrous, the laminae pubescent, the hairs less than 1.0 mm long . . . . . *A. glanduliferum*

Acknowledgments

The first author's research is funded by a grant to Robbin Moran from the United States National Science Foundation (DEB 0717056). The second author thanks Michael Kessler for the invitation to work on the Bolivia Flora Project. We also thank Koos Roux for providing photos and illustrations of *Adiantum poiretii* from Gough Island, Michael Nee for providing details on Otto Kuntze's itinerary in Bolivia, and Layne Huiet and Monica Ponce for helpful comments on the manuscript. The illustrations were prepared by Klei Souza.

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American Fern Journal 100(4):207–218 (2010)

Potential for Spore Germination, Sporophyte Formation and Growth of Young Sporophytes of Four Fern Species from the Atlantic Forest (Brazil)

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ABSTRACT.—The aim of this paper was to study spore germination and growth of young sporophytes of four ferns from Atlantic Forest (Brazil). *Blechnum brasiliense* is a subarborescent fern, *Saccoloma inaequale* is an herbaceous species, while *Cyathea corcovadensis* and *Cyathea delgadii* are tree ferns. The percentages of spore germination under experimental conditions were 65.3 ± 3.6% (*Blechnum brasiliense*), 31.7 ± 2.5% (*C. corcovadensis*), 77.5 ± 5.2% (*C. delgadii*) and 60.5 ± 2.0 (*Saccoloma inaequale*). The highest percentage of sporophytes produced from spore germination was 91.5 ± 2.5% for *B. brasiliense* and the lowest was 35.8 ± 16.2% for *C. corcovadensis*. The relative growth rate (RGR) ranged from 0.17 ± 0.02 cm cm^{-1} $month^{-1}$ (*C. corcovadensis*) to 0.25 ± 0.06 cm cm^{-1} $month^{-1}$ (*S. inaequale*). Spores stored at 7 ± 1 °C for 6 to 8 months were able to germinate and to produce mature gametophyte and sporophytes. The herbaceous species showed greater RGR than the tree species.

KEY WORDS.—germination, relative growth, spore, storage, viability

The world fern flora contains 9,000 to 12,000 species; about 3,250 are found in the American continent (Tryon and Tryon, 1982; Windisch, 2002) and 1,200 to 1,300 species are found in Brazil (Prado, 1997). Sehnem (1977) listed 493 species in the South of Brazil, the majority native to the Atlantic Forest.

According to "Conservation International" the Atlantic Forest is the third diversity "hotspot" in the world and only 8% of its original area remains preserved. In the last few decades, several ferns with ornamental or medicinal proprieties have been indiscriminately exploited and several of them are considered to be endangered species. Examples include some tree ferns belonging to the Cyatheaceae and Dicksoniaceae ("xaxins" or "samambaiaçus"), which have been exploited for commercial purposes, especially in the South of Brazil (Windisch, 2002). *Dicksonia sellowiana* Hook. (Dicksoniaceae) is an endangered tree fern in Brazil due to the extensive harvesting in its habitat (Sehnem, 1978, IBAMA, 1997) and *Rumohra adiantiformis* (Forst.) Ching., (Dryopteridaceae) *Sphaeropteris gardneri* (Hook.) R.M. Tryon, *Cyathea atrovirens* (Langsd. & Fisch.) Domin, *Alsophila setosa* Kaulf, some species of *Adiantum, Asplenium, Blechnum, Selaginella, Marsilea, Adiantopsis* and *Lycopodium* are extracted for ornamental arrangements (Milton & Moll 1988; Windisch, 2002).

According to Pence (2000), the *ex situ* preservation of spores, particularly of rare species from threatened habitats, can be an important supplement to the maintenance of pteridophyte species in the wild. The conditions under which spores are stored have a notable impact on their viability. Generally, to avoid

deterioration, they are stored in dry, ambient or low temperatures, although in some cases this has resulted in loss of viability (Beri and Bir, 1993; Camloh, 1999).

The aim of the present study was to verify the potential of germination of spores stored under refrigeration, the percentage of sporophyte formation after *in vitro* germination, and the relative growth rate (RGR) of young sporophytes cultivated in a growth room of four terrestrial species native from the Atlantic Forest: *Blechnum brasiliense* Desv. (Blechnaceae) an herbaceous species, *Cyathea corcovadensis* (Raddi) Domin and *Cyathea delgadii* Sternb. (Cyatheaceae) both tree ferns and *Saccoloma inaequale* (Kze.) Mett. (Dennstaedtiaceae) an herbaceous fern. These species, with exception of *Saccoloma inaequale,* are routinely used in Brazil as ornamental plants and are exploited indiscriminately from their habitats. *Saccoloma inaequale* is a rare species, which also has potential as ornamental plant. Such information will be useful for management and conservation programs in the future.

Materials and Methods

Blechnum brasiliense and *Cyathea corcovadensis* sporophylls were harvested from several plants on January 12th of 2003; sporophylls of *Cyathea delgadii* were harvested on April 25th of 2002 and of *Saccoloma inaequale* on March 29th of 2003. The collections were carried out in Natural City Park São Francisco de Assis, situated in Blumenau, Santa Catarina State, South Brazil, at 26°55′15″ S and 49°05′30″ W. The study site is a 22.29 ha natural Atlantic forest fragment.

Sporophylls were air-dried in an oven at 30°C for three days on filter paper in order to induce dehiscence. The spores were removed and separated from debris by filtering through lens paper, and stored in glass jars under refrigeration at 7 ± 1°C. Spores of *Blechnum brasiliense* and *Cyathea corcovadensis* were kept under refrigeration for eight months before the germination test; spores of *Cyathea delgadii* and *Saccoloma inaequale* were kept under refrigeration for six months before the germination test. Spores were surface sterilized using a 10% (v/v) solution of commercial bleach (2% of active chlorine) for 20 min. before filtering through sterile filter paper and washing several times in sterile distilled water. Spores of each species were sown in four conical flasks containing 20 mL of Mohr's mineral solution as modified by Dyer (1979) supplemented with Benomyl 0.01%. About 10 mg of spores were inoculated in each flask, which was plugged with two layers of autoclaved transparent commercial polypropylene film (7 × 7 cm) fixed with a rubber band.

All tests were done in a completely randomized design. All procedures were carried out in a laminar hood. Flasks were kept in a growth chamber (25 ± 2°C) under a 16-hour photoperiod and photon flux density of 30 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes. The irradiance was analyzed by a quantameter LICOR 250, of PAR sensor (400 to 700 nm). The percentage of abnormal spores and germination were scored daily or every two days. One

TABLE 1. Analysis of substrate composition (typic hapludult soil with the addition of thermophilic compost in the proportion of 3:1).

Soil composition	
pH	5.2
P (ppm)	+50
K (ppm)	450
Organic matter %	0.9
Al (cmolc/l)	traces
Ca (cmolc/l)	4.8
H^+, Al (cmolc/l)	3.90
N Total %	0.26
CEC (cmolc/l)	12.88

slide from each flask was prepared, with 100 spores counted on each slide. Germinated and abnormal spores were recorded in optical microscopy (40×). Spores were considered to be abnormal when they were totally empty or when they presented a few storage grains between empty spaces. The mean germination time was calculated for each replication per treatment according to the equation:

$$\bar{t} = \sum_{i=1}^{k} n_i t_i \Big/ \sum_{i=1}^{k} n_i$$

where t_i is the time in days starting from day 0 and n_i is the number of spores completing germination on day t_i (Labouriau, 1983; Santana and Ranal, 2004). After one month of cultivation in mineral solution, young filamentous gametophytes were transferred to trays containing sterilized typic hapludult soil (3 parts) with addition of thermophilic organic compost (1 part). The thermophilic compost was obtained from decomposition of vegetable and fruit wastes at the University of Santa Catarina. The substrate (soil with thermophilic compost) analysis was carried out in CIDASC (Companhia Integrada de Desenvolvimento Agrícola de Santa Catarina). The substrate presented a low pH and high levels of P, K, Ca and N (Table 1). The trays were covered with transparent film to avoid excessive water evaporation and plant dehydration. Substrate sterilization was carried out in a high power microwave oven for 20 minutes to avoid contamination with other ferns from the soil spore bank. To analyze the percentage of sporophyte originated from mature gametophytes and to obtain plants that were used later in growth analysis, 200 mature cordate gametophytes were transferred to four trays (50 gametophytes in each tray) containing the same substrate. Gametophytes were separated from each other by 1.0 cm. During the test, gametophytes were watered with sterile distilled water three times a week. The presence of sporophytes was recorded once a week. Plants were kept in a growth room as described above.

When the sporophytes of the four species were big enough to be manipulated (*ca* 3.0 cm tall), they were individually transplanted to small pots containing

TABLE 2. The period of cultivation after spore inoculation for the calculation of RGR of *B. brasiliense*, *C. corcovadensis*, *C. delgadii* and *S. inaequale* in typic hapludult soil with the addition of termophylic compost (3:1) in growth chamber (25 ± 2 C) under a 16-h photoperiod and photon flux density of 30μmol$m^{-2}s^{-}$1. T1 is the day of transplantation to individual pots; T2 is 60 days after transplantation.

	Period of cultivation after spore inoculation (days)	
	T1	T2
B. brasiliense	270	330
C. corcovadensis	337	397
C. delgadii	386	446
S. inaequale	252	312

the same substrate used for gametophyte development and were kept in plastic trays covered with transparent film, in a completely randomized design (Time 1= T_1). For the growth analysis of *Blechnum brasiliense, Cyathea delgadii* and *Saccoloma inaequale,* we utilized 20 sporophytes and for the growth analysis of *Cyathea corcovadensis* we utilized 15 sporophytes. After 60 days of individual transplantation (Time 2= T_2), sporophytes were collected to count the number of fronds and to measure the longest frond's length. Table 2 shows the period of growth after spore inoculation at time 1 and time 2. The relative growth rate (RGR) was estimated according to Bernabe *et al.* (1999), as (Log_{10} L_2 − Log_{10} L_1) / T_2 −T_1 where Log is the base −10 logarithm, L_1 is the initial leaf length when the sporophytes were individually transplanted to the pots in time 1, and L_2 is the leaf length at time 2 (after 60 days of transplantation to the individual pots).

Data were analyzed by Excel for Windows (Microsoft), Minitab for Windows and Statgraphics software. Means and standard deviation were calculated. The Kolmogorov-Smirnov test for goodness of fit for normality and the Bartlett's test for the homogeneity of variance (0.05) were applied before the analysis. The parametric pairwise comparison test among the number of fronds of the four species, were done by the Duncan (5%) Multiple Range Test. The non-parametric Kruskal-Wallis test followed by the Dunn test was employed to analyze the RGR (relative growth rate) because these data did not present residual normality or did not present homogeneity of variance (Santana and Ranal 2004; Zar, 1996). The Student "t" test was applied to compare the number of fronds and the longest frond's length between T1 and T2 for each species,

RESULTS

The mean germination time for the four species studied in this work ranged from 9.9 ± 0.1 days for *Blechnum brasiliense* to 17.8 ± 0.1 days for *Sacolloma inaequale* spores (Table 3). The percentage of germination varied from 32.0 ± 2.5% for *Cyathea corcovadensis* to 77.0 ± 5.0% for *Cyathea delgadii* (Table 3 and Fig. 1a–1d) and the percentage of abnormal spores varied from 5.6 ±

TABLE 3. Days to reach maximum germination (DMG), mean germination time (MGT), maximum percentage of germination (MG), percentage of abnormal spores (AS) and time of spore storage (TS) of *Blechnum brasiliense*, *Cyathea corcovadensis*, *Cyathea delgadii*, and *Saccoloma inaequale* *Cyathea delgadii* Sternb. Spores were sown in liquid Dyer medium in growth chamber (25 ± 2 °C) under a 16-h photoperiod and photon flux density of 30 μmol m^{-2} s^{-1} The table presents data from the literature for comparison. *Germination of unsterilized spore was carried out under continuous white light.

	MGT (Days)	MG (%)	AS (%)	
	DMG (Days)	Mean ± standard deviation		TS (months)
B. brasiliense	15	9.9 ± 0.1	66.0 ± 2.0	29.0 ± 0.7	8
C. corcovadensis	20	15.2 ± 0.5	32.0 ± 2,5	27.1 ± 5.5	8
C. delgadii	18	13.3 ± 0.1	77.0 ± 5.0	14.0 ±1.8	6
S. inaequale	24	17.8 ± 0.1	60.0 ± 1.0	5.6 ± 0.7	6

Data from literature

	DMG (Days)	MGT (Days)	MG (%)	AS (%)	ST (month)	
B. brasiliense	* 5	2,6	60.0	Not analyzed	Fresh spores	Simabukuro *et al.* (1993)
C. corcova-densis	*14	Not analyzed	20.0	Not analyzed	Not informed	Felippe *et al.* (1989)
C. delgadii	*10	Not analyzed	76.0	Not analyzed	Fresh spores	Randi & Felippe (1988 a,b)
C. delgadii	*10	Not analyzed	60.0	Not analyzed	6	Randi & Felippe (1988 a,b)
C. delgadii	*10	5.7	85.0	Not analyzed	Fresh spores	Simabukuro *et al.* (1993)
C. delgadii	*7	Not analyzed	74.0	Not analyzed	12 (at −12 °C)	Simabukuro *et al.* (1998)

0.7% for *Sacolloma inaequale* to 29.0 ± 0.7% for *Blechnum brasiliense* (Table 3).

All species produced sporophytes during the period of analysis, but the percentage of mature gametophytes that produced sporophytes and the time for sporophyte formation were variable among species. *Blechnum brasiliense* presented the first sporophytes after 86 days of cultivation and 91.5 ± 2.5% of gametophytes produced sporophytes after 303 days (Fig. 2a). The first sporophytes appeared in *Cyathea corcovadensis* after 117 days of cultivation and after 264 days of cultivation, 35.8 ± 16.2% of gametophytes produced sporophytes (Fig. 2b). *Cyathea delgadii* presented the first sporophytes after 109 days of cultivation and after 249 days of cultivation, 79.2 ± 13.5% of gametophytes produced sporophytes (Fig. 2c). The first sporophytes of *Saccoloma inaequale* appeared after 78 days of cultivation and after 295 days, 48.4 ± 24.4% of gametophytes produced sporophytes (Fig. 2d).

The number of fronds produced ranged from 4.5 to 7.0 for *Blechnum brasiliense*, from 4.7 to 5.0 for *Cyathea corcovadensis*, from 5.8 to 8.5 for *Cyathea delgadii* and from 6.0 to 9.0 for *Saccoloma inaequale* (Fig. 3a to 3d).

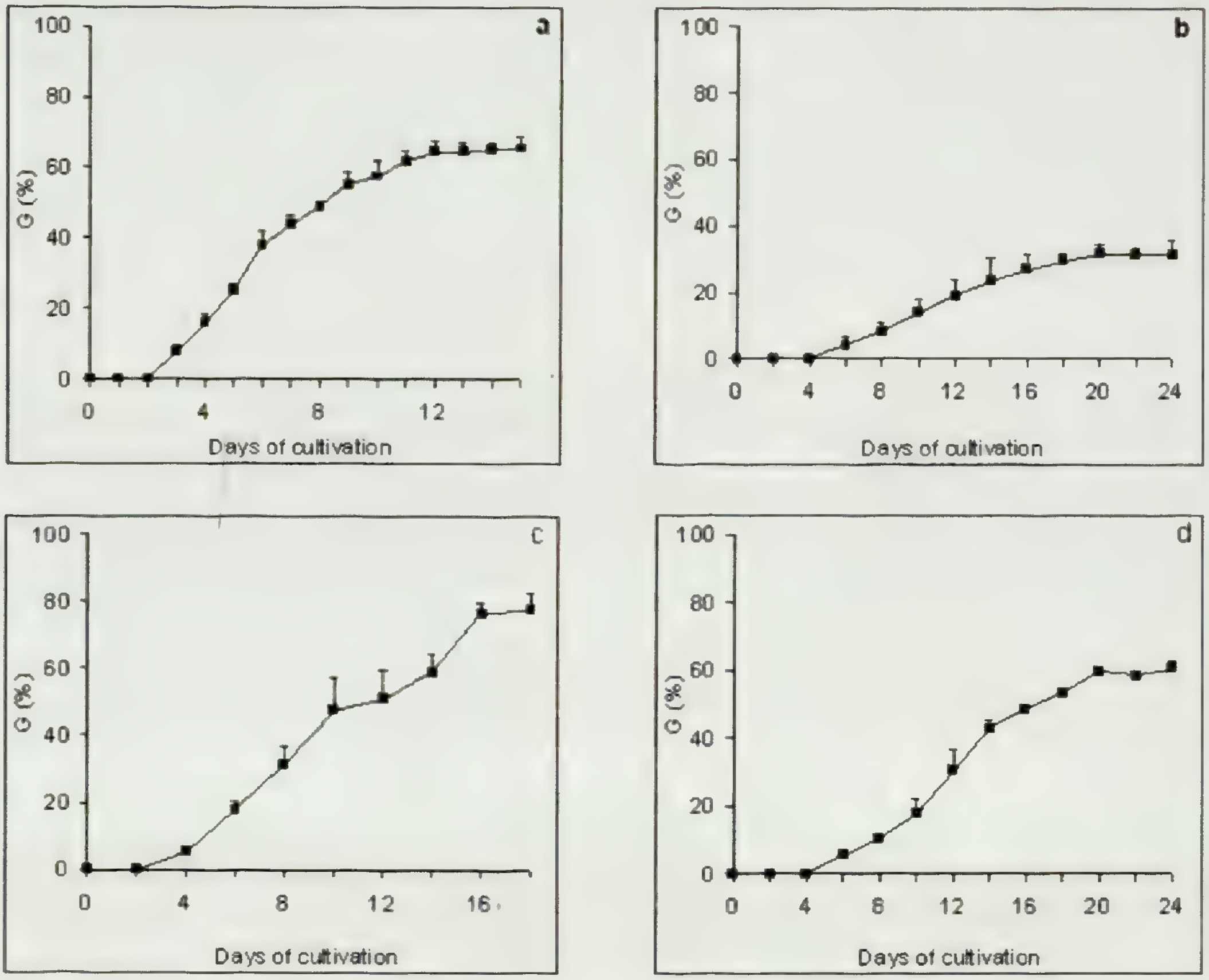

FIG. 1. Germination of spores of *Blechnum brasiliense* (a), *Cyathea corcovadensis* (b), *Cyathea delgadii* (c) and *Saccoloma inaequale* (d) cultivated in liquid Dyer medium in growth chamber (25 ± 2 °C) under a 16-h photoperiod and photon flux density of 30 μmol m^{-2} s^{-1}. Bars are standard deviation. G =germinability.

The longest frond length ranged from 3.0 to 6.9 cm for *Blechnum brasiliense,* from 2.7 to 6.1cm for *Cyathea corcovadensis,* from 4.0 to 10cm for *Cyathea delgadii* and from 3.3 to 9.5cm for *Saccoloma inaequale* (Fig. 4a to 4d). The RGR of *Saccoloma inaequale,* an herbaceous fern, was statistically greater than the RGR of *Cyathea corcovadensis,* a tree fern, but was similar to the other species (Table 4). The number of fronds produced after two months was statistically smaller for *Cyathea corcovadensis* but did not differ among the other species (Table 4).

DISCUSSION

Preservation under refrigeration is a common method of spore storage. According to Pence (2000), the ex situ preservation of spores can be an important supplement to the maintenance of pteridophyte species in the wild. Pence (2000) stored spores of 33 species at 4°C, −20°C, or in liquid nitrogen and observed that spores of three species did not lose viability after more than

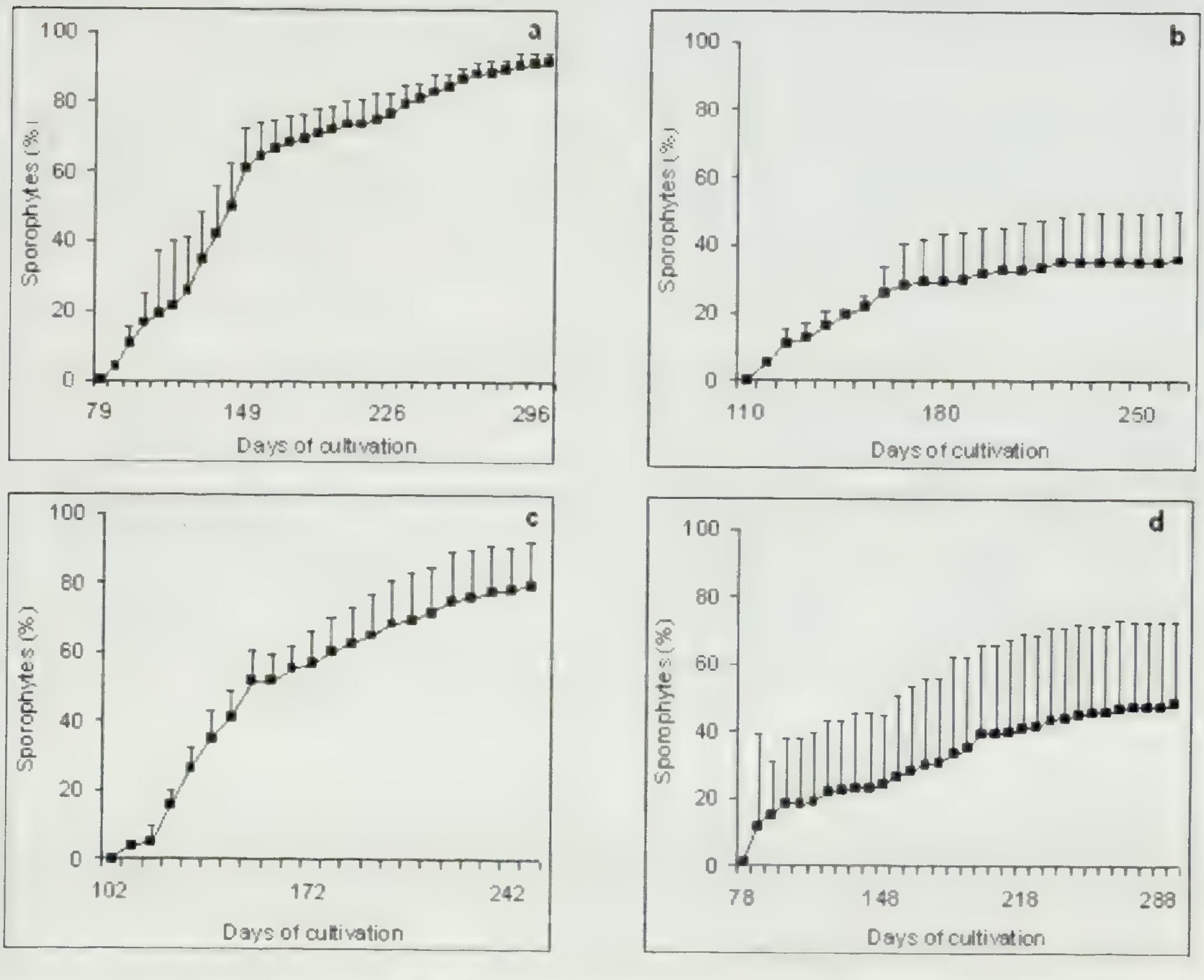

FIG. 2. Sporophyte formation of *Blechnum brasiliense* (a), *Cyathea corcovadensis* (b), *Cyathea delgadii* (c) and *Saccoloma inaequale* (d) cultivated in red soil with the addition of organic compound (3:1) in growth chamber (25 ± 2 C) under a 16-h photoperiod and photon flux density of 30 μmol m^{-2} s^{-1}. Bars are standard deviation.

75 months. Eighty-two percent of *Dicksonia sellowiana* Hook. spores germinated after more than 2 years under refrigeration at 10 C (Filippini *et al.*, 1999). Previously sterilized spores of *D. sellowiana* remained viable after cryopreservation in liquid nitrogen and cryopreservation seems to improve the germination of the spores (Rogge *et al.*, 2000).

Aragon and Pangua (2004) analyzed the spore germination of four rupicolous taxa of *Asplenium* (*A. adiantum-nigrum* L. var. *adiantum-nigrum*, *A. adiantum.nigrum* L. var. *silesiacum*, *A. septentrionale* (L.) Hoffm subsp. *septentrionale* and A. *ruta-muraria* L. subsp. *ruta-muraria*) after 1, 6, and 12 months of storage in Eppendorf tubes (dry storage) or on agar plates (wet storage) at −20°C, 5°C and 20°C. In all cases, except for *A. ruta-muraria*, germination percentage was maintained in wet and dry storage, but in the dry storage method percent germination was higher. Though the authors did not carry out a thorough post-germination study, the plates used in the various experiments remained in culture chambers at 20°C for 6 months and the gametophytes appeared to develop normally. They suggested that storage time might not affect the subsequent development of the prothalli.

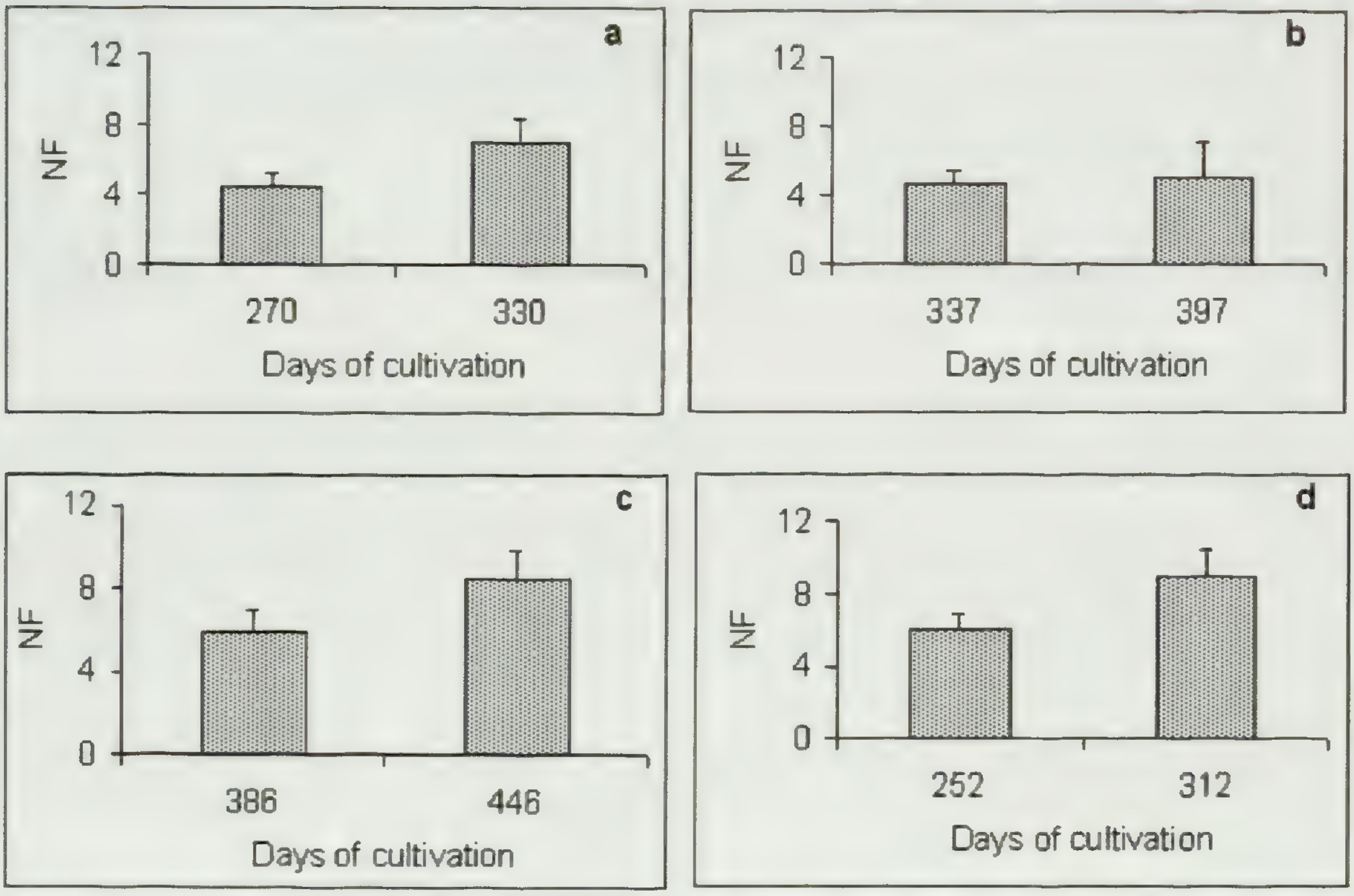

FIG. 3. Number of fronds (NF) of *Blechnum brasiliense* (a), *Cyathea corcovadensis* (b), *Cyathea delgadii* (c) and *Saccoloma inaequale* (d) cultivated in red soil with the addition of organic compound (3:1) in growth chamber (25 ± 2 °C) under a 16-h photoperiod and photon flux density of 30 μmol m^{-2} s^{-1}. Bars are standard deviation.

In comparison to studies done by Simabukuro *et al.* (1993) the mean germination time for spores of *Blechnum brasiliense* and *Cyathea delgadii* were delayed in the present study. However, Simabukuro *et al.* (1993) inoculated fresh unsterilized spores at 25 °C under continuous white light. The surface sterilization could delay or reduce spore germination but is necessary in order to obtain higher percentages of gametophytes. Indeed, Camloh (1993, 1999) reported that the best germination of *Platycerium bifurcatum* (Cav.) C. Chr. occurred when unsterilized spores were used, but that contamination always occurred after 10 days of culture. Simabukuro *et al.* (1998) indicated that before the germination of dry-stored spores, in order to avoid the incidence of fungal growth, there is the need to sterilize them. The differences observed in the present paper for the mean germination time of *B. brasiliense* and *C. delgadii* could be an effect of the time and location of collection, sterilization method for spore germination and time of storage under refrigeration. For *Blechnum chilense* (Kaulf.) Mett. and *Blechnum cycadifolium* (Colla) Sturm, the germination of spores in Thompson agar-gelled medium started after 6–10 days of spore soaking (Pérez-García *et al.*, 1996).

The germination results of stored spores of *Blechnum brasiliense* are similar to the results of Simabukuro *et al.* (1993), who worked with sporophytes of *B. brasiliense* collected in the Biological Reserve of Moji-Guaçu localized in S. Paulo State (southeast region of Brazil). The data presented in this paper are in

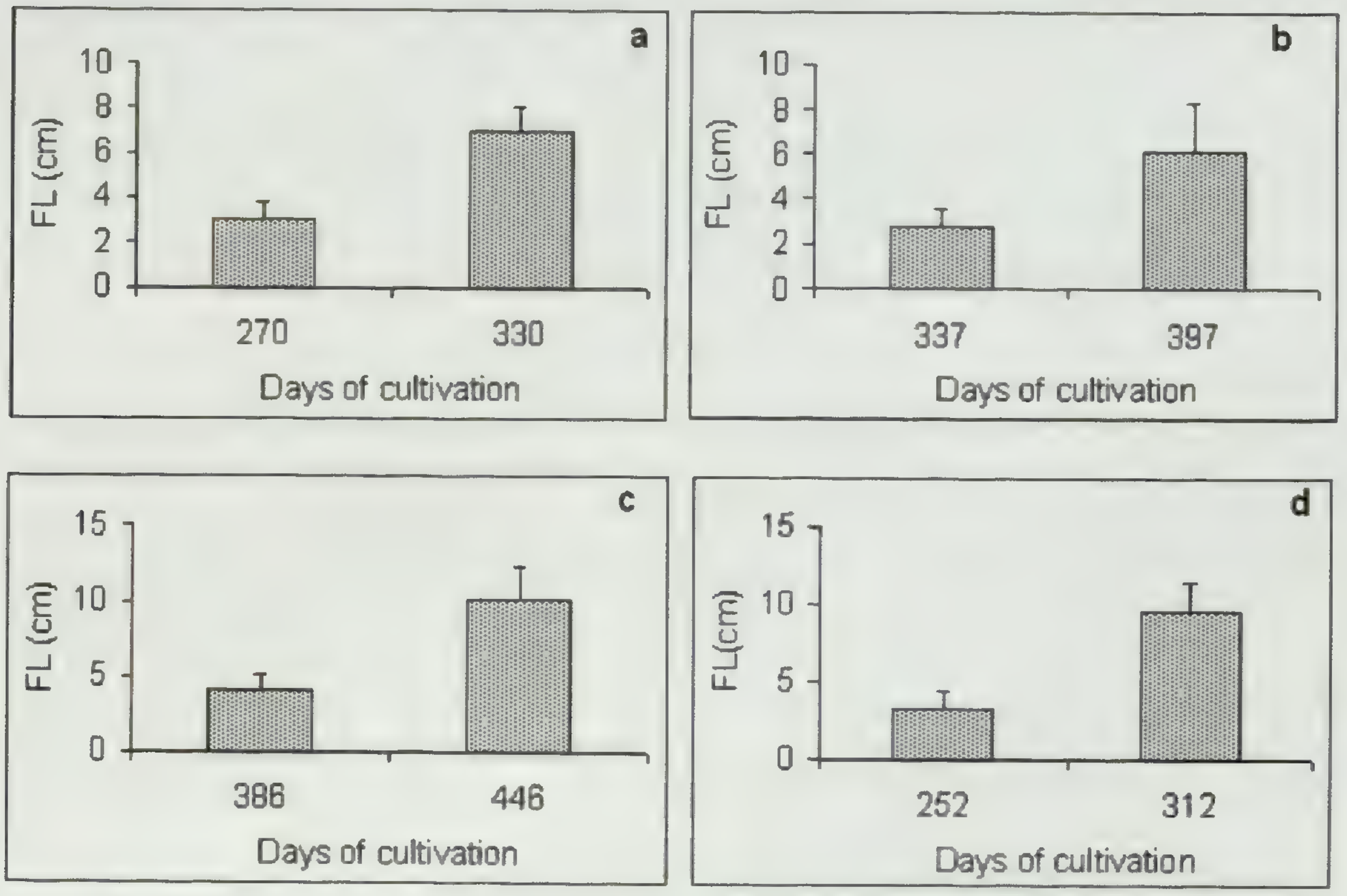

FIG. 4. Longest frond length (FL) of T1 and T2 of *Blechnum brasiliense* (a), *Cyathea corcovadensis* (b), *Cyathea delgadii* (c) and *Saccoloma inaequale* (d) cultivated in red soil with the addition of organic compound (3:1) in growth chamber (25 ± 2 °C) under a 16-h photoperiod and photon flux density of 30 µmol m^{-2} s^{-1}. Bars are standard deviation.

accordance with similar studies in *Cyathea corcovadensis* (Felippe *et al.*, 1989) and *C. delgadii* (Randi and Felippe, 1988 a, b). Simabukuro *et al.* (1998) stored previously sterilized spores of *C. delgadii* for 12 months at −12 C and observed 74% germination after 7 days from sowing. The germination of

TABLE 4. Relative growth rate (RGR) and mean number fronds per month (NF) of *Blechnum brasiliense*, *Cyathea corcovadensis*, *Cyathea delgadii*, and *Saccoloma inaequale* cultivated in red soil with the addition of organic compound (3:1) in growth chamber (25 ± 2 C) under a 16-h photoperiod and photon flux density of 30 µmol m ^{2} s ^{1}. χ^2 is the statistic for the Bartlett test; D_{max} is the statistic for the Kolmogorov-Smirnov test, H is the statistic for the Kruskal-Wallis test; sd is the standard deviation. Letters denote statistical differences. * Data did not show normality.

	B. brasiliense	*C. corcovadensis*	*C.delgadii*	*S. inaequale*
RGR ($cm.cm^{-1}.month^{-1}$) (Mean ± sd)				
	0.18 ± 0.03 ab	0.17 ± 0.02 a	0.19 ± 0.028ab	0.25 ± 0.06 b
χ^2				4.087
D_{max}				0.21*
H				7.12
NF.$month^{-1}$ (Mean ± sd)				
	1.2 ± 0.7b	0.3 ± 0.9a	1.3 ± 0.7b	1.50 ± 0.8b
χ^2				1.259
D_{max}				0.029

sterilized spores of *Saccoloma inaequale* took 24 days. For spores of related *Odontosoria* (Dennstaedtiaceae), germination took six to 15 days to reach maximum percentage (Granados *et al.*, 2003). The abnormal spores observed in this study seems to be related with the reduction in percentage of spore germination for *Blechnum brasiliense* and *Cyathea delgadii*, but it seems to be not the only reason for the low germination of *Cyathea corcovadensis* and *Sacolloma inaequale*.

Large differences in percentages of germination are the rule in simultaneous experiments on different taxa. Spore age, storage conditions and culture conditions, all have a great influence on fern spore germination (Camloh and Gogala, 1992; Camloh, 1993; Camloh, 1999; Sheffield *et al.*, 2001). Differences in the time for sporophyte formation and percentages of sporophyte formation were also common in studies on sporophyte development (Sheffield *et al.*, 2001).

In this study, the first sporophytes of *Blechnum brasiliense* were observed after 2.9 months of cultivation (86 days). Meanwhile, the first sporophytes were observed 4.5 months after spore inoculation for *B. chilense* and after 7 months for *B. cycadifolium* (Pérez-García *et al.*, 1996). The growth of *B. brasiliense* was probably facilitated by the substratum, temperature and photoperiod employed throughout this work. This methodology can then be utilized for the cultivation of *B. brasiliense*. In fact, sporophytes of this species were transplanted to the garden of the Department of Botany and showed a normal development. However, the sporophytes of *C. corcovadensis*, *C. delgadii* and *S. inaequale* were not transplanted to the garden because they showed sensitivity to desiccation and require special cares during acclimatization, needing high humidity, shadow and mild temperatures during this period. *Blechnum brasiliense* appears to be a plant adapted to regimes of oscillating humidity and greater light intensity, being more easily acclimatized.

The gametophytes of *Sacolloma inaequale* that did not produce sporophytes grew successively until the end of this study. A similar observation was found by Sakamaki and Ino (1999) working with *Thelypteris palustris* Schott (Thelypteridaceae). They concluded that sporophytes appeared on most gametophytes that reached a critical size for sporophyte formation, but some gametophytes that did not produce sporophytes became very large and lived for a long time without producing sporophytes.

Our data for frond production by young sporophytes of *Blechnum brasiliense* and *Cyathea delgadii* are similar for the data found for *Acrostichum daneaefolium* Langsd. & Fisch. (Pteridaceae) which were 1.0 ± 0.03 to 1.3 ± 0.04 fronds per month (Mehltreter and Palacios-Rios, 2003; Mehltreter *et al.*, 2003). However, for adult sporophytes of several Cyatheaceae, the number of fronds produced each year varied from 3 to 14 (Tanner, 1983; Ash, 1987; Arens, 2001).

In this study, the RGR of *Sacolloma inaequale* was greater than the RGR of *Cyathea corcovadensis*. Statistically significant differences were not observed among the other species. Data for the RGR of some angiosperms from the Atlantic Forest, presented by Paulilo *et al.* (1993), showed that herbaceous species showed greater RGR than tree species. Concerning RGR for fern

species, data from the literature are very scarce. The RGR for young sporophytes of *Dicksonia sellowiana* cultivated under 10% of natural light was 0.22cm/cm/month (Suzuki *et al.*, 2005), which was similar than data found in the present paper. Bernabe *et al.* (1999) transplanted young sporophytes of *Alsophila firma* (Baker) D.S.Conant (3cm of length) into the interior and at the edge of the forest in Mexico and observed a RGR of 2.42 cm/cm/yr for the first six months of growth in the interior of the forest and 3.53 cm/cm/yr at the forest edge. They concluded that the forest edge where the photosynthetic active radiation (PAR) was nine times higher than the interior of forest was a better place for regeneration of tree ferns. In the present work, PAR at the growth room was very low (30 μmol m^{-2} s^{-1}), which could explain the low RGR found, even for the herbaceous fern.

The results of this study showed that spores stored at 7 ± 1°C for 6 to 8 months, of four species from the Atlantic Forest, were able to germinate *in vitro*, producing mature gametophytes and sporophytes after being transferred to appropriate substrate. The method of spore storage, the germination protocol, and the methods for gametophyte and sporothyte growth employed in this paper can be used by conservation programs for sustainable management and regeneration of these species native for the Brazilian Atlantic Forest.

ACKNOWLEDGMENTS

Áurea Maria Randi thanks CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for the research grant (level 2).

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American Fern Journal 100(4):219–229 (2010)

Studies on Sexual Reproductive Characteristics of *Pteris vittata* L. in Soil Culture

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ABSTRACT.—In order to understand sexual reproductive characteristics of an arsenic hyperaccumulator, *Pteris vittata* L., in soil culture and provide valuable suggestions for its commercial production, two experiments were performed focused on how storage time of spores, collection time, substrate sterilization methods, and culture conditions affect sexual reproductive efficiency. Results demonstrate that spores of *P. vittata* remain at high viability after several month storage, can live through hot water and spores collected in winter have a higher viability than those collected in spring. Furthermore, light is indispensable for gametophyte growth of *P. vittata.* During the gametophyte phase and the early sporophyte phase, *P. vittata* demonstrated a wide adaptability to differing light intensities, but grew best under 40% shade. Additionally, it grew in sand though it prefers fertile substrates. A proper combination of light, substrate, and densities of spores and gametophytes promises efficient reproduction.

KEY WORDS.—collection time, light, *Pteris vittata*, sexual reproduction, spore viability, storage time, substrate

Pteris vittata L., commonly known as Chinese brake fern, is an arsenic hyperaccumulator, as well as a potential ornamental fern. Despite its great value in phytoremediation and gardening, it is not used widely. Numerous studies have been performed on this species. The mechanism of uptake and accumulation of arsenic by *P. vittata* has been studied in detail (Nie, 2006; Wang *et al.*, 2007; Yang *et al.*, 2007; Wei, 2008; Zheng *et al.*, 2008); its characteristics of gametophyte development have been documented (Zeng, 2001); and several regeneration methods, such as tissue culture and spore sterile culture, have been studied (Xu, 2006; Shi, 2008). However, much remains unknown, especially an understanding of the optimal conditions for the growth of gametophytes and sporophytes of *P. vittata* in soil culture. Compared with tissue culture and spore sterile culture, soil culture is simpler, cheaper and more convenient (Jiang, 2001), which could enhance the commercial production of *P. vittata*. Therefore, in order to understand its sexual reproductive characteristics in soil culture, two experiments were performed focused on how collection time, storage time of spores, substrate

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TABLE 1. Spore germination and gametophyte growth of the sowing group. The earliest green time was the earliest time when the substrate surface turned green after sowing; the earliest mature time was the earliest time when sexual organs emerged after sowing; the earliest time for juvenile sporophytes was the time when the first juvenile sporophyte emerged after sowing. The mean number of juvenile sporophytes of each replicate was recorded on 3rd September, 2008, when most juvenile sporophytes had at least three fronds.

Treatment	Collection dates	Sowing dates	The earliest green time*	The earliest mature time*	The earliest time for juvenile sporophytes*	Mean number of juvenile sporophytes (per replicate)
S1	2007.11.15.	2008.3.19.	14	62	74	120
S2	2008.1.16.	2008.3.19.	17	40	55	100
S3	2008.3.13.	2008.3.19.	17	62	101	37

* The number of days after sowing.

sterilization methods, and culture conditions affect the sexual reproductive efficiency of *P. vittata* in soil culture.

MATERIALS AND METHODS

Experiment I

Experiment I was conducted in an artificial climate box, aiming to reveal how collection time of fertile fronds, storage time of spores, and substrate sterilization with boiling water affect the sexual reproductive efficiency of *P. vittata*. Two groups, a sowing group and a no sowing group, were set. The no sowing group was set to examine sterilization efficiency. Based on three different collection times, three treatments (marked S1, S2, and S3) with four replicates per treatment were set in the sowing group. The no sowing group also included three subgroups (marked N1, N2, and N3) with four replicates in each subgroup. Therefore, there were 24 replicates in total. The experimental procedures are as follows:

Spore collection.—Fertile fronds of different plants of *P. vittata* were collected on three different days (Table 1) in South China Botanical Garden (Guangzhou, China), wrapped in paper bags, and dried at room temperature for four days to release spores. Then spores were collected in envelopes and stored at 4°C until used.

Substrate preparation.—Twenty-four transparent plastic cups of 6 cm in diameter with holes in the bottom were filled with sand, leaving space from the surface of sand to the cup mouth. After the sand was sterilized with boiling water, cups were sealed with plastic film to keep the sand away from pollution. Holes in the bottom of each cup helped prevent oversaturation.

Sowing and tending.—Spores weighing 0.05 g for each replicate of the sowing group by an electronic balance (Sartorius BS124S) were wrapped with filter paper, then sterilized using 1000× dilution of 50% Carbendazim powder and washed with hyperpure water three times, then made into a suspension.

Once the suspension was dropped onto the surface of sand evenly with a burette, the cup was sealed instantly with plastic film. No spores were sown in the no sowing group. After sowing, samples of the two groups were put in a tray filled with water. Then they were put in the artificial climate box. The cultivation conditions were: 18 $\mu mol \cdot m^{-2} \cdot s^{-1}$ fluorescent light for 15 hours, 25 °C, and 85%RH; dark for 9 hours, 20 °C, and 90%RH.

Observations.—To study the condition of spore germination and gametophyte development, random samples were taken weekly from the sowing group. Spores and gametophytes were mounted in water and observed under a light microscope (Zeiss Axioplan 2). Several parameters were recorded: the earliest green time, which was the earliest time when the substrate surface turned green after sowing; the earliest mature time, which was the earliest time when sexual organs emerged after sowing; the earliest time for juvenile sporophytes, which was the time when the first juvenile sporophyte emerged after sowing. The number of juvenile sporophytes of each replicate was counted when most juvenile sporophytes had at least three fronds. Meanwhile, quality of juvenile sporophytes was noted including the number and the size of fronds, and the number of pinnae. For the no sowing group, only the number of juvenile sporophytes of each replicate was recorded.

Experiment II

Experiment II was conducted in a plastic house, focused on how light and substrate affect the sexual reproductive efficiency of *P. vittata*. Five levels of sun light intensity and three kinds of substrate were laid out as follows: no shade (A), 40% shade (B), 85% shade (C), 96% shade (D), and dark (E), accomplished by different covers of black shade netting; sand (a), mixture of 50% sand and 50% farm soil (b; v/v), and mixture of 50% peat soil, 25% sand, and 25% farm soil (c; v/v). There were 15 treatments in total, with four replicates per treatment. The experimental procedures are as follows:

Spore collection.—Fertile fronds of *P. vittata* were collected on 23rd March, 2008 in the same place as Experiment I (Table 1), wrapped in paper bags, and dried at room temperature for four days to release spores. Then spores were collected in envelopes and stored at 4°C until used.

Substrate preparation.—Three kinds of substrate were put in 60 plastic garden pots of 12 cm in diameter, then sterilized with 500× dilution of Dexon powder and sealed as Experiment I for a week. Each tray under the pot was filled with water to ensure substrate inside was moist. One week later, plastic film was taken away and substrate was exposed directly to air for one week in order to let poisonous gas produced by the dilution of Dexon powder release gradually.

Sowing and tending.—Spores were weighed to 0.2 g for each replicate by an electronic balance (Sartorius BS124S) and wrapped with newspaper. After spores were sowed evenly on the substrate surface, the pot was sealed immediately with plastic film. Trays under the pots were kept full of water during this experiment. Meanwhile, microclimate condition under the five

different light treatments including temperature, relative humidity, and sun light intensity, was detected by a humidity-temperature meter (TES1364) and a light meter (TES1335) respectively at 2:30 to 3:00 in the afternoon every three or four days after sowing. Spores were sown on 18th April, 2008.

Observations.—Inspection of the condition of spore germination and gametophyte development with naked eyes occurred weekly. The number of replicates with at least 50% area green, the earliest time for juvenile sporophytes, and the number of juvenile sporophytes of each replicate were recorded in detail. For convenience, the number of juvenile sporophytes of each replicate was counted when the first juvenile sporophyte emerged. The earliest green time was recorded but not discussed in this experiment because algae or mosses turned the substrate surface green instead.

Results

Experiment I

Effect of storage time and collection time on the sexual reproductive efficiency of P. vittata.—The time when the substrate surface turns green is considered as the time when spores germinate here. According to the collection time and the sowing time (Table 1), storage times of spores were about four months, two months and one week, respectively. Spores stored longer did not germinate later. Spores with the longest storage time (S1) germinated earlier than those in S2 and S3, though the difference was not obvious. The substrate surface of S2 and S3 turned green simultaneously, though spores in the former was collected two months before sowing, and those in the latter collected just one week before sowing. Additionally, a longer storage time did not delay occurrence of sexual organs and juvenile sporophytes definitively. Sexual organs and juvenile sporophytes arose earliest in S2, but latest in S3. However, the longer spores were stored, the more juvenile sporophytes were born. The largest number of juvenile sporophytes was detected in S1, but only 37 juvenile sporophytes were found in S3 (Table 1), which was about one third of S2. In terms of collection time, spores collected in winter, such as on November 15th, 2007 and January 16th, 2008, germinated earlier and yielded more offspring than those collected in spring on March 13th, 2008.

Storage time and collection time together appear to influence the quality of juvenile sporophytes. Except S2, every replicate in S1 and S3 was covered fully with juvenile sporophytes. In addition, juvenile sporophytes from S1 or S3 were nearly the same size. In S1, sporophytes of each replicate had four to seven fronds with no more than two pairs of pinnae and the longest frond grew up to 3 cm long. In S3, sporophytes of each replicate had no or one to three fronds with no pinna and the longest frond grew up to 2 cm long. However, in S2 some sporophytes of one replicate had seven fronds while those of the remaining three replicates only had one to four fronds.

TABLE 2. Effect of light on the sexual reproductive efficiency of *P. vittata*. A, no shade; B, 40% shade; C, 85% shade; D, 96% shade; E, dark. The number of replicates with at least 50% area green was recorded 30 days after sowing; the earliest time for juvenile sporophytes was the time when the first juvenile sporophyte emerged after sowing; the total number of juvenile sporophytes of each treatment was recorded when the first juvenile sporophyte emerged.

Treatment	Number of replicates with at least 50% area green	The earliest time for juvenile sporophytes*	Total number of juvenile sporophytes
A	4	32	5
B	9	32	60
C	9	32	9
D	7	52	4
E	3	/**	0

* The number of days after sowing; ** no juvenile sporophytes emerged until 18th August, 2008.

Relationships among the earliest green time, the earliest mature time, and the earliest time for juvenile sporophytes.—Turning green earlier did not lead to an earlier occurrence of sexual organs. The sample in S2 became mature 22 days earlier than the other two treatments (Table 1), but S2 was not the first to turn green. S1, which turned green first, shared the same earliest mature time with S3, which turned green three days later than S1 (Table 1).The earlier sexual organs emerged, the earlier juvenile sporophytes arose. However, S1 and S3 did not produce sporophytes at the same time, though their sexual organs were born simultaneously.

Efficiency of sterilization with boiling water and its effect on spore viability.—Boiling water did not kill all spores in sand. No algae or mosses were detected in the sowing group or no sowing group. However, in one replicate each of N1 and N2, there were a small number of sporophytes born in N1 and N2 (N1 = 4 juveniles; N2 = 7 juveniles). Most sporophytes found in the no sowing group were recognized as *P. vittata*, and the rest died before they were big enough to be recognized.

Experiment II

Effect of light on the sexual reproductive efficiency of P. vittata.—Spores germinated under five levels of light intensity, even though those in the dark still germinated. However, the largest numbers of replicates with at least 50% area green were achieved under 40% shade and 85% shade. The earliest time for juvenile sporophytes was the same for treatment A, B and C, while sporophytes emerged 20 days later under 96% shade and no sporophytes were detected in the dark (Table 2). When the first juvenile sporophytes emerged, the largest number of juvenile sporophytes was achieved under 40% shade, 51 more than that under 85% shade (Table 2).

Effect of substrate on the sexual reproductive efficiency of P. vittata.—Spores germinated in three kinds of substrate. The largest number of replicates with at least 50% area green was achieved in the mixture of 50% peat soil, 25% sand,

TABLE 3. Effect of substrate on the sexual reproductive efficiency of *P. vittata*. a, sand; b, mixture of 50% sand and 50% farm soil; c, mixture of 50% peat soil, 25% sand, and 25% farm soil. The number of replicates with at least 50% area green was recorded 30 days after sowing; the earliest time for juvenile sporophytes was the time when the first juvenile sporophyte emerged after sowing; the total number of juvenile sporophytes of each treatment was recorded when the first juvenile sporophyte emerged.

Treatment	Number of replicates with at least 50% area green	The earliest time for juvenile sporophytes*	Total number of juvenile sporophytes
a	8	52	52
b	9	32	19
c	15	32	7

* The number of days after sowing.

and 25% farm soil, while only eight replicates with at least 50% area green in sand. Sporophytes arose simultaneously in substrate b and c, but 20 days later in sand (Table 3). However, the largest number of juvenile sporophytes was detected in sand rather than substrate b and c. Substrate c, the richest substrate, yielded the fewest juvenile sporophytes.

Comprehensive effect of light and substrate on the sexual reproductive efficiency of P. vittata.—Replicates in combination Aa, Ea, and Eb did not achieve 50% area green, and all treatments under 40% shade and 85% shade had the largest number of replicates with at least 50% area green. The earliest time for development of juvenile sporophytes differed among treatments. Sporophytes developed earliest in some combinations, such as Ab, Ac, Bb, Bc, Cb, and Cc; those in combinations Ba and Db developed later, and no sporopohytes were detected in combinations Da, Ea, Eb, and Ec. Combination Ba yielded the largest number of juvenile sporophytes, 43 more than combination Bb (Table 4).

DISCUSSION

Green time and germination time.—In general, when a rhizoid emerges, the spore germinates (Zhang and Niu, 1999; Xu *et al.*, 2005; Xu, 2007). Based on our observation, when the gametophyte of *P. vittata* was only two to three cells long, chloroplasts were easily observed under the light microscope. In other words, the time when the substrate surface turns green is close to the time when spores germinate. Thus green time of the substrate surface is a reasonable and convenient indication of germination time.

Spore viability.—Results of Experiment I demonstrate that spores of *P. vittata* remain at high viability after several month storage and can live through hot water. High spore viability and strong heat-resistance ability of spores may contribute to the wide distribution of *P. vittata*, which spreads in the tropical areas and subtropical areas in Asia and America, occupies almost 25% area of China and even reaches Shaan'xi Province and Gansu Province, Northwest of China. Its distribution is growing. In 2006, for the first time, natural

TABLE 4. Comprehensive effect of light and substrate on the sexual reproductive efficiency of *P. vittata*. A, no shade; B, 40% shade; C, 85% shade; D, 96% shade; E, dark. a, sand; b, mixture of 50% sand and 50% farm soil; c, mixture of 50% peat soil, 25% sand, and 25% farm soil. The number of replicates with at least 50% area green was recorded 30 days after sowing; the earliest time for juvenile sporophytes was the time when the first juvenile sporophyte emerged after sowing; the total number of juvenile sporophytes of each treatment was recorded when the first juvenile sporophyte emerged.

Treatment	Number of replicates with at least 50% area green	The earliest time for juvenile sporophytes*	Total number of juvenile sporophytes
Aa	0	/**	0
Ab	1	32	4
Ac	3	32	1
Ba	3	52	51
Bb	3	32	8
Bc	3	32	1
Ca	3	72	1
Cb	3	32	4
Cc	3	32	4
Da	2	/**	0
Db	2	52	3
Dc	3	59	1
Ea	0	/**	0
Eb	0	/**	0
Ec	3	/**	0

* The number of days after sowing; ** no juvenile sporophytes emerged until 18th August, 2008.

populations of *P. vittata* were found on the city wall built in the Ming Dynasty, Nanjing, the capital of Jiangsu Province, East China. *P. vittata* was never reported in Jiangsu Province previously (Li, 2008). High spore viability, strong heat-resistance ability of spores, and wide distribution of *P. vittata* makes it a candidate for inclusion in gardening and phytoremediation.

It is commonly known that the longer storage time is, the later spores germinate (Pan *et al.*, 2007). However, in Experiment I, a longer storage period of spores did not delay germination and formation of sexual organs and sporophytes of *P. vittata*. This is distinct from the study on *Osmunda japonica* Thunb., which showed that given the same sowing density, spores stored longer tended to geminate later and led to later formation of gametophytes and sporophytes (Deng, 2004). This is because spores of the two genera are different. Spores of *Osmunda* have chlorophyll while those of *Pteris* do not, and the spore coat of *Osmunda* is so thin that its spores tend to dry out but the spore coat of *Pteris* is thick enough to resist drying.

Data from Experiment I shows that spores of *P. vittata* collected in winter have a higher viability than those collected in spring. This may be due to the increased activity of microbes in the spring. It is reported that microbes can reduce viability of spores and obstruct formation of gametophytes and sporophytes (Simabukuro, 1998). Microbes are rich under natural conditions. Spores used in Experiment I were exposed to microbes during the process from

TABLE 5. Sun light intensity, relative humidity and temperature under five different light levels.

Treatment	Sun light intensity ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Relative humidity (%)	Temperature (°C)
A	217.587±154.9299	88.25769±9.9522	29.96154±3.131242
B	158.1323±85.7517	84.31923±11.7896	30.56154±3.453324
C	37.02845±22.91277	83.71538±12.65758	30.43077±3.442245
D	10.89365±7.42368	84.96538±13.11077	30.09615±3.402542
E	0	87.61538±10.71748	30.03462±3.365771

fertile frond collection to spore collection, so some spores may have been contaminated by them before sterilization and thus had low viability. Due to the cold and dry weather in winter, spores collected during that time might be less affected by microbes than those collected in the warm and wet weather of spring. This could be the reason that spores of *P. vittata* collected in winter geminated earlier and yielded more offspring than those colleted in spring. Therefore, collecting spores in dry and cold weather ensures a high viability of spores, especially for *P. vittata*. Additionally, in order to kill microbes effectively before sowing, proper sterilization methods for spores and substrate are necessary.

Growth habits of gametophytes and juvenile sporophytes.—Data from Experiment II indicates that light is indispensable for gametophyte growth of *P. vittata*. However, spores could germinate in the dark in Experiment II. Spores of *Woodwardia radicans* (L.) Sm. also germinated in the dark during wet storage at 20 °C (Quintanilla *et al.*, 2002), but spores of *Osmunda cinnamomea* L. var. *asiatica* Fern. did not germinate in the dark (Jiang, 2001). This could be associated with chlorophyll. As mentioned above, spores of *Osmunda* have chlorophyll while those of *Pteris* and *Woodwardia* do not. Thus spores of *Osmunda* need light to germinate, while those of the other two genera do not.

Results of Experiment II imply that at the gametophyte phase and the early sporophyte phase, *P. vittata* has a wide adaptability of light intensity, but it grew best under 40% shade and most gametophytes under no shade died before the birth of sporophytes. This suggests that adequate shade is necessary for the well being of gametophytes of *P. vittata* despite being a hardy fern that likes brightness. Indeed many ferns need careful tending, similar to *P. vittata* at the gametophyte phase and the early sporophyte phase (Kong, 2000; Huang, 2006).

Different levels of light intensity lead to different sexual reproductive efficiencies of *P. vittata*. This is strongly connected to the microclimate under different covers of black shade netting. Combining the data about sun light intensity, relative humidity and temperature (Table 5) and the total number of juvenile sporophytes of each treatment (Table 2), a conclusion can be drawn that gametophytes and juvenile sporophytes of *P. vittata* prefer bright conditions with high moisture and mild temperature.

Besides a wide adaptability of light intensity, *P. vittata* also has a strong adaptability from poor substrate to rich substrate and could regenerate itself by spores in sand. This is why it can be found on sidewalks, building crevices, and nearly every habitat with exposed limestone and forms to several populations on the city wall in Nanjing (Li, 2008). However, it seems to prefer fertile substrate as the most gametophytes developed in the mixture of peat soil, farm soil and sand in Experiment II. Thus rich soil is preferable for a high reproductive efficiency of *P. vittata*.

When the first juvenile sporophytes arose in Experiment II, the largest number of juvenile sporophytes was achieved in substrate poor in nutrition, such as sand, rather than substrate rich in nutrition, such as the mixture of 50% peat soil, 25% sand, and 25% farm soil. This might be connected with the time counting the number of juvenile sporophytes. Given enough time, the number of juvenile sporophytes in substrate rich in nutrition would probably increase and even surpass that in substrate poor in nutrition. Therefore, a more reasonable time should be selected to count the number of juvenile sporophytes and more parameters should be set to examine effect of substrate on sexual reproductive efficiency of *P. vittata*. The physical structure of substrate could influence the number of juvenile sporophytes as well (Pan *et al.*, 2007). Given that the substrate was always saturated with water in Experiment II, due to the loose structure in sand, there might be more liquid water in sand than in the mixture of peat soil, farm soil and sand, so sperm of gametophytes cultured in sand could reach archegonia more easily. This is consistent with the conclusion that liquid water is critical for ferns at the gametophyte phase in sexual reproduction though nutrition is badly needed after sporophytes are born (Bao *et al.*, 2000; Sheffield *et al.*, 2001). Therefore, a mixture of sand and other materials in a proper proportion and enough fertilizer are indispensable for a high efficiency of sexual reproduction of ferns.

Besides culture conditions, densities of spores and gametophytes are factors that determine an optimal number of juvenile sporophytes. For instance, in Experiment II the largest number of replicates with at least 50% area green was achieved in the mixture of 50% peat soil, 25% sand, and 25% farm soil 30 days after sowing (Table 3), but the smallest number of juvenile sporophytes was found in those same conditions. Besides the improper time counting the number of juvenile sporophytes and the physical structure of the mixture, a density of spores or gametophytes that was too high may have resulted in lack of nutrition, an unbalanced proportion of antheridia and archegonia or something else (Liu and Liu, 2001), though a higher density of gametophytes contributes to more antheridia and more sporophytes for some species (Raghavan, 1989; Pan *et al.*, 2007). Further study should be performed to reveal how densities of spores and gametophytes affect the number of juvenile sporophytes and what proper densities of spores and gametophytes are for the commercial production of *P. vittata*.

The information provided in this paper certainly will be useful for the commercial production of *P. vittata*. However, further study remains to be performed as our study is focused on the early growth of *P. vittata*.

ACKNOWLEDGMENTS

We gratefully acknowledge the grant received from the science and technology project of Shenzhen City, Guangdong Province, China.

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American Fern Journal 100(4):230–233 (2010)

A New Status for the Dwarf Maidenhair Fern of the Pacific Northwest Coast

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ABSTRACT.—The dwarf maidenhair fern of the Pacific Northwest coast is here recognized as a variety of the western maidenhair fern; that is, as *Adiantum aleuticum* var. *subpumilum*. Reasons are given for this treatment.

KEY WORDS.—*Adiantum aleuticum*, taxonomy, dwarf maidenhair

Along the Pacific Coast of Vancouver Island, British Columbia, grows a variant of the northern maidenhair fern (*Adiantum pedatum* L.) complex that differs from regionally typical material of mesic forest habitats by its much shorter stature and imbricate pinnules. This dwarf coastal form was described as *Adiantum pedatum* L. var. *subpumilum* W.H. Wagner (Wagner and Boydston, 1978). It was considered a variety of *A. pedatum* because western North American plants were considered conspecific with those in eastern North America where the type is from. This dwarf coastal form has been known in cultivation since the 1950's, but the original collection locality was kept secret by its discoverer(s), and wild populations were not re-discovered until 1977 (Wagner and Boydston, 1978). At present the dwarf coastal form is known from four wild populations along the western coast of Vancouver Island, from 48°38′N to 50°10′N.

Paris (1991) separated the western North American representatives of the *Adiantum pedatum* complex (as well as a subset of populations from eastern North America) from the nomenclaturally typical form as *Adiantum aleuticum* (Rupr.) Paris. This was justified by subtle morphological distinctions and isozyme differences between the two taxa. Paris, however, did not recognize any infraspecific taxa in *A. aleuticum*. She believed that morphological differences among populations of *Adiantum aleuticum* were not consistent or predictable, and that results of chloroplast and DNA studies of *A. aleuticum* showed limited genetic divergence among populations (Paris 1991a, b). For that reason, infraspecific taxa within *Adiantum aleuticum* were not recognized in the *Adiantum* treatment for Flora of North America (Paris 1993). Because the *subpumilum* form is apparently a variant of *Adiantum aleuticum*, if *subpumilum* were to be recognized as an infraspecific taxon, it should be treated as a variety or subspecies of *A. aleuticum*, not *A. pedatum*. The need for this treatment was noted previously by Ceska (2000), but he did not make the new combination.

The dwarf coastal form of *Adiantum aleuticum* apparently has a genetic basis for its dwarfed morphology (Olsen, 2007). For over 50 years it has been

maintained in cultivation by propagation from spores. During this time it has kept its smaller stature, suggesting it is not a mere environmental variant. Furthermore, the dwarf coastal form of *A. aleuticum* differs from other morphological variants of this species with imbricate pinnules, particularly those found on ultramafic substrates such as serpentine, with its small fronds and plane (rather than upright) pinnae. In regard to inland variants, such as those described by Cody (1983), most are associated with serpentine and other ultramafic substrates. *Adiantum aleuticum* is in fact a strong serpentinophile in the Pacific Northwest (Kruckeberg, 1969). Variation in the morphology of serpentine forms appears to be, at least in part, a function of the degree of exposure to full sun. For example, plants growing in shady serpentine habitats are extremely vigorous but otherwise not significantly deviant from the morphology exhibited by plants of *A. aleuticum* growing in mesic non-ultramafic habitats. However, variation in *A. aleuticum* throughout its range is deserving of more detailed morphological and genetic study.

There might be an adaptive advantage to the shorter stature exhibited by the dwarf coastal maidenhair fern. Full size fronds of *A. aleuticum* growing on coastal cliffs, where they are exposed to salt laden spray and strong winds, often die back at the distal portions of the pinnae. Genetic mutations that shorten the length of the pinnae could provide a selective advantage in habitats that are exposed to salt spray. These are exactly the habitats in which the wild populations of the dwarf coastal maidenhair fern are found (pers. obs.).

Because the dwarf coastal form of the northern maidenhair fern propagates true from spores, and is known from multiple natural populations in a specific specialized habitat (i.e., coastal cliffs subject to salt spray), it should be recognized as a valid taxon. Recognition of the dwarf coastal maidenhair as a valid taxon, regardless of rank, is important to protect wild populations under private and government biodiversity protection programs. Designation as a variety of *Adiantum aleuticum* rather than a subspecies seems warranted because var. *subpumilum* is essentially geographically sympatric but ecologically allopatric with *Adiantum aleuticum* var. *aleuticum*. For these reasons, the transfer of the dwarf coastal maidenhair fern to *Adiantum aleuticum* is proposed herein:

Adiantum aleuticum (Rupr.) Paris var. *subpumilum* (W.H. Wagner in Wagner & Boydston) E.R. Alverson, **comb. nov.**

Adiantum pedatum L. var. *subpumilum* W.H. Wagner, Canad. J. Bot. 56:1727. 1978. *Adiantum pedatum* L. ssp. *subpumilum* (W.H. Wagner) Lellinger, Amer. Fern J. 74:62. 1984. TYPE.—CANADA, **British Columbia**, Brooks Peninsula, NW Vancouver Island, small drainage system S of Orchard Point, 50°10′ N, 127°50′ W, 27 June 1977, *J. Pojar and F. Boas 770191* (holotype MICH; isotypes V and UBC).

Treatment of the dwarf coastal form of *Adiantum aleuticum* as a variety is warranted because it is a well marked, genetically based morphological variant associated with specific selective habitat parameters under natural conditions.

Further morphological and genetic study of *Adiantum aleuticum* var. *subpumilum* is warranted. The full geographic distribution of this variety has yet to be documented. While populations of var. *subpumilum* occur along much of the length of the west coast of Vancouver Island, similar coastal habitats to the north and south of Vancouver Island should also be searched. For example, populations of *Adiantum aleuticum* occur on coastal cliffs just south of La Push, Clallam County, Washington. It is not clear that the reduced pinna length exhibited by some of these populations has the same genetic basis as Vancouver Island populations of var. *subpumilum* (E. Alverson, pers. obs.). Populations of *Adiantum aleuticum* also occur on coastal bluffs in Oregon, but I have not observed Oregon populations that approach the morphology of var. *subpumilum*. However, these coastal habitats can be extremely difficult and dangerous to access, and further field surveys may provide additional data on the distribution of var. *subpumilum*.

Coastal habitats in British Columbia and the adjacent regions of the Pacific Coast are known for a sizable number of endemic taxa that suggest a significant history of geographic isolation and Pleistocene refugia (Douglas, 1996). *Adiantum aleuticum* var. *subpumilum* is just one of a longer list of vascular plant taxa, including *Enemion savilei* (Calder & Tayl.) Keener, *Geum schofieldii* Calder & Taylor, *Ligusticum calderi* Mathias & Const., *Lloydia serotina* (L.) Reichenb. ssp. *flava* Calder & Taylor, *Saxifraga taylori* Calder & Savile, *Senecio moresbiensis* (Calder & Tayl.) G.W. Dougl. & G. Ruyle-Dougl., *Trillium ovatum* Pursh var. *hibbersonii* Taylor & Szczawinski, and *Viola biflora* L. ssp. *carlottae* Calder & Taylor, that are associated with putative Pleistocene refugia on Vancouver Island and adjacent regions. While most if not all of Vancouver Island was covered by glacial ice during the most recent glacial advance, it is possible that these endemic taxa survived the glacial maximum on the unglaciated coastal margin that was exposed due to lower sea levels, but is now below the present sea level. If *Adiantum aleuticum* var. *subpumilum* is indeed a Pleistocene relict, it may have diverged from *Adiantum aleuticum* var. *aleuticum* before the current Holocene climatic optimum. Further studies of DNA variation could cast more light on the genetic and evolutionary history of *Adiantum aleuticum* var. *subpumilum*.

Acknowledgments

I thank two anonymous reviewers for helpful comments on an earlier draft of this paper.

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American Fern Journal 100(4):234–237 (2010)

Shorter Notes

New Fern Records for the State of Zacatecas and San Luis Potosi, Mexico.—Based on recent collections made in the last two years, we have found two species that are recorded for the first time from the states of Zacatecas and San Luis Potosi, Mexico. Our taxonomic and distributional standard has been the Mexican pteridoflora by Mickel and Smith (Mem. New York Bot. Gard. 88:686–692. 2004), which includes both a general range statement and the state-level distribution of each taxon based on cited voucher specimens.

Mickel and Smith (2004) treated eight species of the genus *Woodsia* as occurring in Mexico. The genus is widely distributed in north temperate regions and higher elevations in the tropics. *Woodsia phillipsii* Windham was previously known from Arizona, New Mexico and Texas in USA, and is widely distributed in Mexico, from Baja California Norte, Baja California Sur, Chihuahua, Coahuila, Durango, Jalisco, Mexico, Michoacan and Sonora, but has not been documented previously from Zacatecas.

This species is characterized by stramineous stipe bases, linear to narrowly oblong blades, translucent projections on pinnule margins mostly multicellular, often to form filaments, indusial lobes filamentous most of their length and far exceeding the sporangia and 7–18 pairs of pinnules. Our specimens match the description except for the spores, which are 45–50 μm bigger than those described. Mickel and Smith (2004) discuss these plants with larger spores as possible allotetraploid hybrids.

The voucher specimens of the *Woodsia phillipsii* population were collected at Municipio Jerez de García Salinas, 2 km from El Cargadero Dam, 22°43′N, 103°06′W (*Pacheco et al., 3843* UAMIZ; *Pérez-Jaramillo et al. 55* UAMIZ). The plants were growing in secondary *Quercus* forest at 2331 m. The population occupies a small area, but the plants are abundant.

On the other hand, *Didymoglossum petersii* (A. Gray) Copel. is known from southeastern United States to Costa Rica, but in Mexico has only been reported from Chiapas, Puebla and Veracruz. The documented distribution is likely biased because of the small size of the plants that has resulted in under collection of the species by botanists. Frequently it grows on the walls of calcareous sinkholes at 1400–2200 m.

Didymoglossum petersii is related to *Didymoglossum ovale* E. Fourn. in its small size, the lack of submarginal veins and the presence of stellate marginal hairs, but differs in its lack of dark involucral margins.

Didymoglossum petersii was discovered in San Luis Potosí, Municipio Xilitla, Manantial de San Antonio, 21 22′N, 99 00′W (*Pacheco et al., 3886* UAMIZ; *Sánchez-Morales et al. 244* UAMIZ). The tiny plants were growing as trunk epiphytes on the north side of the stream in wet montane forests at 1140 m.—Leticia Pacheco and Andrés Sánchez-Morales, Universidad Autónoma Metropolitana-Iztapalapa, Depto. de Biología, Área de Botánica Estructural y Sistemática Vegetal. Apdo. Postal 55-535, 09340 México, D. F., México.

Notes on the Natural History and Reproductive Biology of *Isoëtes malinverniana*.—*Isoëtes malinverniana* Cesati *et* De Notaris is a narrowly endemic species from the administrative districts of Lombardy and Piedmont in north-west Italy. To date only twelve populations of this plant grow in a heavily disturbed habitat in the plain of the river Po. Water channels where this plant lives are under the constant threat of nutrient enrichment and pesticide contamination deriving from their use as water supply for rice crops. This is one of the main reasons for the species was listed as Critically Endangered in the Italian IUCN Red-List (Conti *et al.*, Università di Camerino. 1997) and is presently under study for the aims of conservation.

Isoëtes malinverniana is a tetraploid species ($2n = 44$; Löve *et al.*, Cytotaxonomical atlas of the Pteridophyta. J. Cramer, Vaduz. 1977) of uncertain origin that is genetically distant from all the other species in the genus. The closest relatives are some species (i.e., *I. coromandelina* L.f.) from the south-east Asia (Hoot *et al.*, Syst. Bot. 31:449–460. 2006). Here we give some insights on the reproductive biology and phenology of *I. malinverniana* as a result of field and laboratory observations for the sake of addressing future conservation efforts.

In 2010, germination tests were run with *I. malinverniana* spores at the Lombardy Seed Bank of the University of Pavia (LSB), with the aim to test for the self-compatibility of the species. Since *I. malinverniana* is very rare, the experiment was limited to two plants, to avoid affecting the survival of the source population which is located in a branch of the Cavo Bogino Channel, a few hundred meters north of the town of Vigevano, in the Lombardy district. Specimens used as a source of spores in this study were cultivated at the Botanical Garden of the University of Pavia.

Megaspores from the two plants were sown on 1% agar after wetting the surface of the substrate with sterile water. A total of 134 megaspores (64 and 70 from plant A and B, respectively) were plated into two different Petri dishes. Megaspores from plant A were fertilized adding microspores from plant A and the same procedure was followed for plant B using its own microspores. Spores were incubated at 20° C in light (12/12h day/night) and checked weekly for the emergence of sporelings. The experiment ended after four weeks when all the megaspores had germinated.

Sporophyte production was rapid, resulting in 100% and 99% emergence from megaspores from plants A and B, respectively, within the fourth week. These reproductive rates are very high when compared with other *Isoëtes* species in culture. Depending on the treatment, Kott and Britton (Can. J. Bot. 60:679–1687. 1982) obtained on average 87–89% megaspores emitting sporophytes for *I. macrospora* Durieu, 58–63% for *I. tuckermanii* A.Braun, 2–39% for *I. riparia* Engelm., 0–17% for *I. acadiensis* Kott and 0–15% for *I. echinospora* Durieu.

Our results seem to rule out the self-incompatibility of this species reported by Schneller (Webbia 35: 307–309. 1982), who noted that in nature megasporangia of *I. malinverniana* mature well before microsporangia and that this lack of synchrony is expected to increase the chance of cross

fertilization. Our observations, gathered in the field during the whole year between 2008 and 2009 on wild populations and cultivated plants revealed that in *I. malinverniana* development of microsporangia precedes that of megasporangia. The latter were present from January to June and localized in the innermost leaves. Some mature megaspores were already visible at the end of January. Microsporangia were localized in the outermost leaves and matured earlier in both wild and cultivated plants starting from the end of December onward. Our observations are in contrast with other authors who reported that spore dispersal for *I. malinverniana* begins between summer and autumn (Pignatti, Edagricole Bologna. 1982; Marchetti, Ann. Mus. Civ. Rovereto 19:71–231. 2003). A possible explanation for this discrepancy may be found in the possibility to find some mature megaspores until October. Despite the high viability of the spores, sporelings of *I. malinverniana* are short-lived in laboratory and they often die in the first three months. Even in the field, the observation of young plants is very rare. The causes are still unknown, but probably involve both genetic and ecological factors. In some sites the water flow was very fast and can be a cause of up-rooting of the sporelings.

Eighteen sporelings grown in an aquarium very close to the mother plant were dug immediately after their emergence to determine if they were the product of sexual or vegetative reproduction. The presence of a megaspore at their base excluded a vegetative origin from the mother plant, but we cannot exclude apogamy. Vegetative reproduction is known in *I. tegetiformans* Rury (Rury, Amer. Fern J. 68:99–108. 1978), *I. storkii* T.C.Palmer (Gomez, Brenesia 18:1–14. 1980), *I. lechleri* Mett. (Hickey, Fieldiana (Bot) NS 34:88–97. 1994), *I. andicola* (Amstutz) L.D.Gómez (Karrfalt, Amer. Fern J. 89:198–203. 1999) and *I. sinensis* Palmer (Chen *et al.*, Biodiversity Science 12:564–571. 2004), but to date, no evidence of this ability has been observed in *I. malinverniana*.

Our results provide new and important insights in *I. malinverniana* ecology. Firstly, we have more clearly elucidated the reproductive phenology of this species showing that megaspores and miscrospores develop during the winter, through the spring and until the early summer. Through culture experiments, we have also found that megaspores germinate promptly and rapidly produce sporophytes, and are highly self compatible, a likely event given the contemporary presence of both kinds of spores.

Recent AFLP and ISSR analysis (Gentili *et al.*, Aquat. Bot. 93:147–152. 2010) revealed high genetic variability within *I. malinverniana* populations in comparison to other species in the genus. According to the authors this could be a consequence of the out-breeding behavior of the species and of its high level of ploidy. In light of our results, the latter explanation seems more important for the maintenance of genetic variability and it could also play a role in enforcing the viability and germination ability of the spores (Kott and Britton, 1982).

The rapid emergence of the sporelings we obtained at 20°C, suggest that *I. malinverniana* megaspores do not require a cold stratification to germinate. Incubation at low temperatures has been reported to increase germination in

other species such as *I. echinospora*, *I. riparia* and *I. acadiensis* (Kott and Britton, 1982). In *I. malinverniana*, however, recruitment of megaspores through winter stratification deserves confirmation and new experiments aimed to test cold stratification in cultured spores are ongoing.—THOMAS ABELI, DISTA, Dipartimento di Scienze della Terra e dell'Ambiente, University of Pavia, via S. Epifanio 14, 27100, Pavia, Italy, e-mail: thomas.abeli@unipv.it, and MARCO MUCCIARELLI, Dept. of Veterinary Morphophysiology, University of Torino, via Leonardo da Vinci 44, 10095, Grugliasco (TO), Italy, e-mail: marco.mucciarelli@unito.it.

American Fern Journal 100(4):238 (2010)

Referees for 2010

All papers submitted to the journal are peer reviewed. Members of the editorial board and the American Fern Society, as well as additional scientists in cognate areas do these reviews on a voluntary basis. It is their work that contributes to the high quality of articles in the *American Fern Journal* and to its continued success. The American Fern Society and I extend our thanks to the following reviewers for the assistance, diligence, and patience in the year 2010 (I apologize if I inadvertently omitted anyone from this list).

Safaa Al-Hamdani
Todd Aschenbach
David Barrington
Elke Bellefroid
James Caponetti
Subhash Chandra
David Conant
Laura Cornara
Don Farrar
Natalia Fernandez
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Publication title and number: *American Fern Journal* (0002-8444). Date of filing: September 26, 2010. Frequency of issue: quarterly. Annual subscription price 2010: $25.00. Office of Publisher: c/o Missouri Botanical Garden, 4344 Shaw Blvd., St. Louis, MO 63110-2225. Editor: Jennifer Geiger. The *American Fern Journal* is wholly owned by the American Fern Society, Inc., with no bond holders. Physical address of the Society: c/o Missouri Botanical Garden, 4344 Shaw Blvd., St. Louis, MO 63110. The purpose, function, and nonprofit status of the Society and its tax exempt status for Federal income tax purposes remains the same as in past years. The average press run for Volume 100 is 850, and was 850 for the issue appearing immediately prior to the filing date, for which 761 copies were mailed as paid circulation and 0 copies were mailed as free distribution, leaving 89 copies for office use and back-issues sales. I certify that these statements are correct and complete. George Yatskievych, Membership Secretary of AFS.

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Volume 100, number 3, July–September, pages 137–187, issued 4 January 2011

Volume 100, number 4, October–December, pages 189–244, issued 19 April 2011

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